RESISTANCE TO EMAMECTIN BENZOATE IN SEA LICE

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ABSTRACT

Effective control of parasitic sea lice (including *Lepeophtheirus salmonis* and *Caligus elongatus*), is a major challenge currently facing sea cage salmon aquaculture. Emamectin benzoate (EMB; SLICE®), a macrocyclic lactone (ML), has been the drug of choice for sea lice treatment over the past decade due to its ease of administration, as well as efficacy, on all parasitic stages of the salmon parasite. This over-reliance led to increased tolerance to the drug and a consequent decline in its use. Macrocyclic lactone resistance has been linked to ATP-binding cassette (ABC) transporters such as P-glycoprotein (*P-gp*) in arthropods and nematodes. The present study investigated the role of *P-gp* in sea lice resistance to EMB. Analysis of bioassay results indicated a 4 to 26 fold higher EMB EC_{50} for *L. salmonis* samples collected from salmon farms in the Bay of Fundy, New Brunswick (NB), in 2011 compared with a similar study carried out between 2002 and 2004, suggesting loss of EMB efficacy in sea lice in this region. An assay for ATPase activity (utilizing SB-MDR1-S*f*9 membranes over-expressing *P-gp*), as well as a competitive inhibition test, showed that EMB interacts with this transporter. Emamectin benzoate caused a significant concentration-dependent increase in P-gp mRNA expression in *L. salmonis*. Also, results indicated a temporal increase in P-gp mRNA levels in archived *L. salmonis* collected from 2002 to 2011. These findings suggest that EMB is a substrate for *P-gp*, will induce higher expression of the transporter, and that the latter is likely involved in loss of EMB efficacy in *L. salmonis*. Contrary to initial prediction that prior host immunostimulation would enhance EMB efficacy in the parasite, sea lice attached to salmon with a history of immunostimulation exhibited greater survival and significantly higher P-gp mRNA expression compared with control groups ($P < 0.05$). Prior host immunostimulation

likely caused increased expression of P-gp which could have consequently caused decreased EMB efficacy.

The present study also investigated (using bioassays and RT-qPCR) reports of differences in sea lice EMB sensitivity in salmon farming regions in the Bay of Fundy. *Lepeophtheirus salmonis* collected from Grand Manan [Bay Management Area (BMA) 2b] recorded >2 fold lower EMB EC_{50} values compared with BMAs 1 and 2a populations, confirming the presence of relatively EMB-sensitive sea lice in BMA 2b. Laboratory-reared sea lice maintained their EMB sensitivity status for up to three filial generations. *Caligus elongatus*, collected from BMA 2a, also showed >2 fold lower EMB EC_{50} values compared with *L. salmonis* collected from the same site, indicating species differences in sensitivity to the parasiticide. Attempts to localize sea lice P-gp protein and mRNA *in situ* using immunohistochemistry and *in situ* hybridization techniques, respectively, were not successful. Similarly, the quantification of *P-gp* in *L. salmonis* using ELISA and Western blot techniques was unsuccessful. This could be due to low P-gp expression at mRNA and protein levels and/or poor antigenic specificity of the antibodies used.

Results in this study strongly support the hypothesis that the efflux transporter, *P-gp*, is involved in reduced sensitivity of *L. salmonis* to EMB, and that this occurs over time due to drug selection of resistant strains of the parasite. However, there is a need to develop sensitive tools, e.g. probes and antibodies, which will localize and quantify *L. salmonis* P-gp mRNA and protein production. This will allow for a greater understanding of the role of *P-gp* in sea lice resistance to EMB as well as a greater ability to monitor resistance development to the parsiticide. Changes in the expression of resistance-associated genes, such as those for P-gp, can be monitored and used in the diagnosis of resistance development to parasiticides. Knowledge of

the timing for resistance development will inform necessary changes to treatment options to prevent treatment failure. Further comparisons between EMB-sensitive and -resistant strains of *L. salmonis* may be necessary to verify the degree to which *P-gp* is involved in the loss of parasite sensitivity to the drug. More investigations on the nature of the species differences in EMB sensitivity in sea lice, as well as the heredity of EMB resistance mechanisms, is necessary for a better understanding of how this parasite strives to survive its control.

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List of Abbreviations

ABC	ATP-binding cassette
AChE	Acetylcholinesterase
AVC	Atlantic Veterinary College
Avr	Avermectin
BBB	Blood-brain-barrier
BMA	Bay Management Area
BSA	Bovine serum albumin
CAHS	Centre for Aquatic Health Sciences
Cdc37	Cell division cycle 37
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CLR	C-lectin receptor
CpG ODN	Cytosine-phosphate-guanine oligodeoxynucleotide
DAB	3, 3'-Diaminobenzidine
DIG	Digoxigenin
dpS	Days post-commencement of emamectin benzoate treatment
dpSC	Days after cessation of emamectin benzoate treatment
EC_{50}	Half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
eEF1α	Translation eukaryotic elongation factor 1α
ELISA	Enzyme-linked immunosorbent assay
EMB	Emamectin benzoate
FAO	Food and Agriculture Organization

GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GluCl	Glutamate-gated chloride channel
GlyR	Glycine receptor
HcGluCl	*Haemonchus contortus* GluCl
HRP	Horseradish peroxidase
IC_{50}	Half-maximal inhibition concentration
IL	Interleukin
IVM	Ivermectin
LB	Lysogeny broth
LGIC	Ligand-gated ion channel
Log k_{ow}	Octanol-water coefficient
MANOVA	Multivariate analysis of variance
MDCK	Madin-Darby canine kidney
MDR	Multi-drug resistance
MH	Major histocompatibility
ML	Macrocyclic lactone
MMP	Matrix metalloproteinase
NBT/BCIP	Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate
NMDA	*N*-methyl-D-aspartic acid
PBS	Phosphate buffered saline
P-gp	P-glycoprotein (protein)
P-gp	P-glycoprotein (gene/mRNA)

P_i	Inorganic phosphate
ppb	Parts per billion
ppt	Parts per thousand
Rdl	Resistance to dieldrin
RNAi	RNA interference
RPS20	Ribosomal protein S20
RT-qPCR	Reverse transcription quantitative PCR
SSC	Saline-sodium citrate
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TSK	Trimmed Spearman-Kaber
USEPA	US Environmental Protection Agency

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Salmon Aquaculture

Aquaculture, which has seen tremendous growth and diversification over the years (FAO 2012), was first documented by the Chinese in 475 BC (Liao & Chao 2009). The increase seen in fish farming is partly due to the realization that fish and other aquatic species are cost-effective and healthier sources of animal protein compared with land-based livestock. Atlantic salmon, *Salmo salar*, is one of the most intensively farmed marine fish (Naylor & Burke 2005) and its commercial production is currently dominated by the aquaculture industries of Norway, Chile, Scotland, Ireland, and Canada (Boxaspen 2006, Lees et al. 2008).

The demand for salmon has been rising over the years and, as with any such enterprise, as demand increases, there is pressure to produce more as well. As with other sectors of aquaculture, the steady growth in commercial salmon production comes with increasing challenge to the industry (Olesen, Myhr & Rosendal 2011). Challenges to aquaculture (such as salmon farming) include, but are not limited to infections, animal welfare, environmental perturbation, and ecological impacts (Buschmann et al. 2009, Burt et al. 2012). Of these challenges, the most significant are viral, bacterial, fungal and parasitic infections, which can manifest as outbreaks on salmon farms (Asche et al. 2009). In addition to direct costs incurred by salmon farmers for disease treatments, secondary challenges of disease management and control include negative consequences such as antibacterial resistance in humans (Midtlyng, Grave & Horsberg 2011) and toxicity to benthic non-target species of economic and ecological significance (e.g. lobster) (Burridge et al. 2010).

Of the numerous infections that may affect salmon, sea lice have become one of the most prominent. This is mainly due to current difficulty in controlling the parasite on salmon farms. However, the greatest financial losses due to sea lice are attributed to reduced growth rate and

poor feed conversion ratio (Mustafa, Rankaduwa & Campbell 2001). An estimate of sea lice costs to the world salmonid farming industry was generated by Costello (2009), using data from countries where sea lice have been reported to be a problem. The analysis assumed that sea lice control costs increased in proportion to production. Multiplying the sea lice costs by the Food and Agriculture, Fisheries and Aquaculture Information and Statistics Service salmonid production figures for 2006, indicated a total cost of CAD $496 million. As profit margins get narrower because of increased competitiveness, increased fish-feed costs, and pressure on farms to control sea lice to prevent spread to wild fish and other farms; sea lice control remains a significant challenge to commercial salmon production.

Salmon aquaculture not only suffers from the direct cost associated with sea lice treatments and reduced market value of affected salmon, but also negative public image, particularly during major sea lice outbreaks (Hansen & Onozaka 2011). Although sea lice infection on wild salmon have been documented for several decades (White 1940, Berland & Margolis 1983, Margolis & Berland 1984), declines in wild salmon populations have been blamed on the presence of high numbers of sea lice in commercial sea cage salmon farms (Anderson, Whoriskey & Goode 2000, Krkošek et al. 2011, 2013). However, factors affecting sea lice epizootics in the ocean, both for the farmed and wild salmonids, are numerous and can be very complex (Frazer 2009).

1.2. Sea lice

Sea lice are ectoparasites known to cause mortalities in wild and farmed salmonids (White 1940, Bakke & Harris 1998, Todd et al. 2000). *Lepeophtheirus salmonis* and *Caligus elongatus* are the major species of sea lice that infect Atlantic salmon, *S. salar,* in Atlantic Canada and Northern Europe, *L. salmonis* and *C. clemensi* in Pacific Canada, while *C.*

rogercresseyi is a significant pest in Chile (Burka, Fast & Revie 2012). *Lepeophtheirus salmonis* and *C. clemensi* are the sea lice species infecting salmonids on the Pacific coast of Canada. Recent studies have suggested that Pacific *L. salmonis* differ from Atlantic *L. salmonis* in terms of genetic make-up (Todd et al. 2004, Yazawa et al. 2008), host pathogenicity (Saksida et al. 2007a) and infection of alternate hosts such as three spine stickleback (Jones & Prosperi-Porta 2011).

Sea lice have a direct life cycle (Fig. 1.1) with attached juveniles and mobile preadult and adult stages on the host (Schram 1993). *Lepeophtheirus salmonis* and *C. elongatus* undergo 10 and 8 developmental stages, respectively, during their life cycle, each separated by a molt. The first 2 stages are planktonic; the third, the copepodid, is the first infective stage that locates and attaches to the host and is frequently found on skin, gills and other external surfaces on the host; the 4 chalimus stages attach to the salmon host by a frontal filament, while the last 3 stages (2 preadults and an adult in *L. salmonis*; no preadult stages in *C. elongatus*), often referred to as 'mobiles', can move freely on the surface of the host and are the stages that are most pathogenic to the host (White 1942, Johnson & Albright 1991). The mobile stages can transfer from one host to another and may be critical where single year class smolt stocking is not practiced (Ritchie 1997).

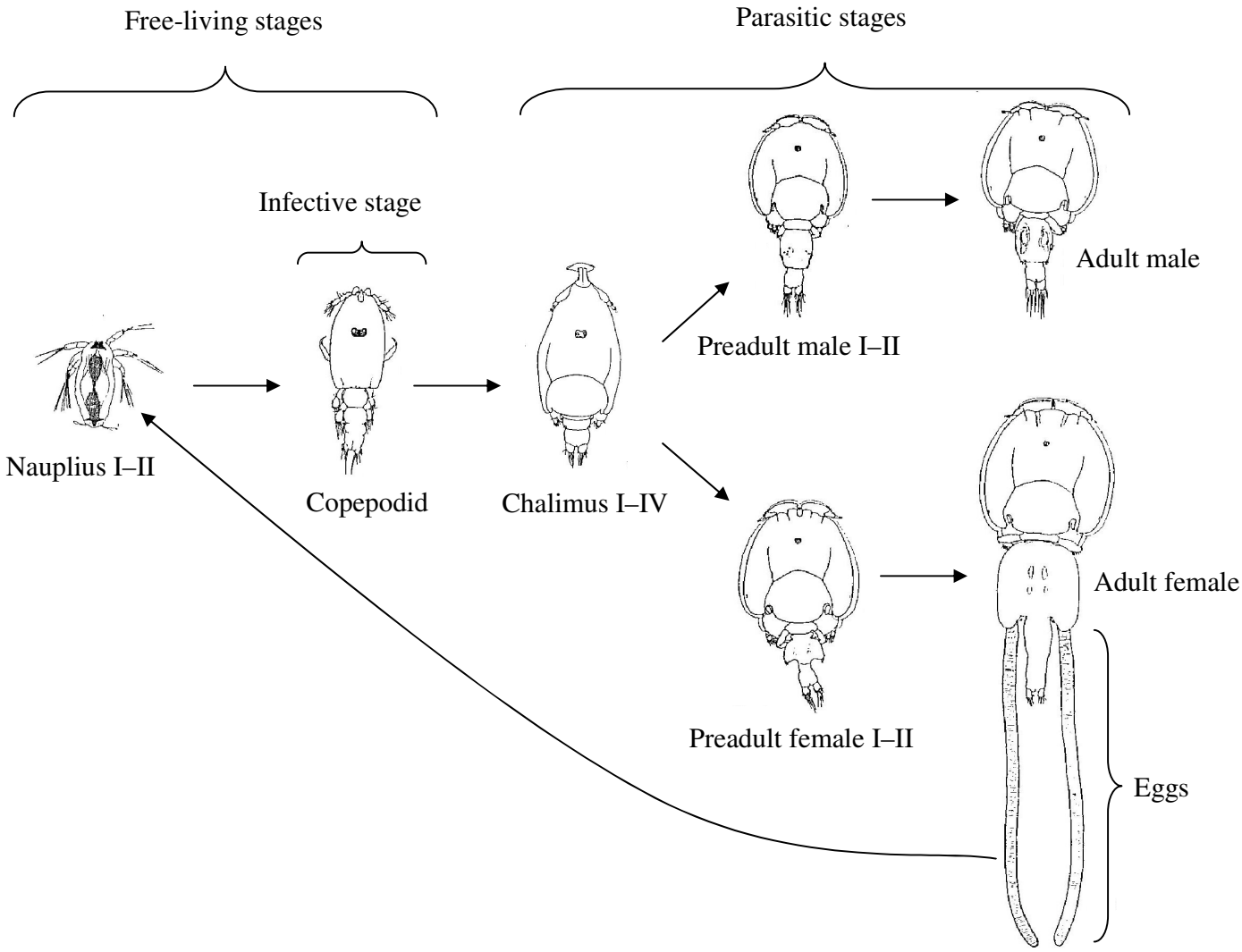

Figure 1.1. Developmental stages of *Lepeophtheirus salmonis* [diagram (not to scale) modified from Schram 1993].

1.2.1. Factors affecting sea lice survival and dispersal

According to Bron et al. (1991), *L. salmonis* copepodids prefer to attach to the fins and mostly locate the host salmon through chemoattractants (Ingvarsdóttir et al. 2002) and taste cues originating from the host (Pert et al. 2009). Local water current (Bron et al. 1991), temperature, salinity (Heuch, Nordhagen & Schram 2000, Bricknell et al. 2006) and light intensity (Genna, Mordue & Pike 2005) have all been shown to affect survival and settlement of sea lice copepodids. For instance, poor settlement was recorded at ~7°C compared with 12°C; and at 24 ppt salinity compared with 34 ppt (Tucker, Sommerville & Wootten 2000). Also, at low water temperature (7.1°C), sea lice egg production, hatchability and development are reduced compared with 12.2°C (Heuch, Nordhagen & Schram 2000), suggesting that temperature may be an important factor in the biology of early stages of the parasite. Earlier work by Johnson and Albright (1991) showed that as water temperature decreased from 15 to 5 °C, development time of eggs increased and, that at salinity of 10 and 15 ppt, there were neither egg nor nauplii development, respectively. Copepodids in this latter work were obtained only at 30 ppt.

Sea lice infections on wild salmonids were reported several decades ago (White 1940). Although there is literature stating that salmon farms may contribute to sea lice epizootics in wild salmon populations (Krkošek et al. 2013), how the contribution relates to declines in wild salmonids (Parrish et al. 1998, Levin & Schiewe 2001) is not well understood. It is most likely that cross-infection of sea lice occurs between farmed and wild hosts. In Pacific Canada, refuge for sea lice includes farmed and wild salmon (Saksida et al. 2007b, Marty, Saksida & Quinn 2010), opportunistic hosts or alternate nearshore environments where they overwinter and are then available to directly infect new hosts or to release larvae in late winter (Brooks 2009).

Contrary to the situation in Pacific Canada, in Atlantic Canada, knowledge of sea lice refuge and/or where the parasites overwinter are largely lacking.

The first outbreaks of sea lice infection occurred on Atlantic salmon farms during the 1960s and late 1980s in Norway and North America, respectively, soon after the introduction of sea cage salmon aquaculture (Pike & Wadsworth 1999). Confinement of large numbers of salmon in sea cages creates an ideal environment for sea lice to easily locate a host and multiply rapidly (Bakke & Harris 1998). The entire age and development range is frequently present within the salmon cage, although the stage population depends on the type and effectiveness of control measures in place (Pike & Wadsworth 1999).

1.2.2. Pathology and host responses to sea lice infections

Host response to sea lice infection depends on several factors including species of host as well as species and stage of the parasite. *Lepeophtheirus salmonis* is larger, more aggressive and more pathogenic than *Caligus* spp. Host responses to an infection of sea lice range from depressed appetite to death (Boxaspen 2006). Newly attached copepodids of *L. salmonis* cause a local cellular response on the skin which can be seen as an inflamed dark spot visible to the naked eye. Low-level *L. salmonis* infection (8-11 sea lice per fish) caused several changes in the expression of the following immune genes in the Atlantic salmon: major histocompatibility (MH) class I, MH class II and interleukin (IL) 1β (Fast et al. 2006), but did not cause remarkable stress responses (Tully & Nolan 2002, Fast et al. 2006). However, some studies reported increased stress in Atlantic salmon in response to a higher (~100 per fish) sea lice burden (Bowers et al. 2000, Mustafa et al. 2000) and was attributed to development through to late chalimus, and preadult stages. Contrariwise, 10 sea lice per fish for up to 10 days caused increased Na^+/K^+-ATPase and chloride ion concentrations compared with 3 and 6 sea lice/fish

groups (Nolan, Reilly & Wendelaar Bonga 1999) indicating stress to the fish. This indicates that as the number of sea lice on a fish and infection period increase, the level of stress on the fish increases.

Other problems associated with sea lice infection include edema, sloughing of the scales and epidermal pathology, such as necrosis of skin pavement cells, apoptosis of inner epidermal cell layers and increased mucus discharge (Nolan, Reilly & Wendelaar Bonga 1999). Abrasion of the skin predisposes the host to secondary bacterial and viral infections (Jónsdóttir et al. 1992, Barker et al. 2009) as well as osmoregulatory problems (Wagner et al. 2003). Parasite-induced increase in irritability may also lead to changes in behaviour including poor swimming performance (Wagner et al. 2003) and flashing.

1.2.3. Control of sea lice

Several strategies have been used or are being developed for the control of sea lice in salmon farms [(Rae 2002) Table 1.1] and include biological predators (Bjordal 1990, Deady, Varian & Fives 1995), vaccines (Raynard et al. 1994, Grayson et al. 1995, Raynard et al. 2002, Ross et al. 2006, Frost, Nilsen & Hamre 2007, Carpio et al. 2011), immunostimulants (Covello et al. 2011, Purcell et al. 2012, Poley et al. 2013), drugs and chemicals (Burka et al. 1997, Burka, Fast & Revie 2012), as well as good management practices such as disinfectants (Pietrak & Opitz 2004), fallowing, single year class, and removal of sick or dead salmon (Bron et al. 1993). Freshwater bath treatment was not successful in controlling the parasite (Stone et al. 2002). Differences in susceptibility to sea lice infection within Atlantic salmon families (Glover, Nilsen & Skaala 2004, Glover et al. 2005, Gjerde & Saltkjelvik 2009) can also be exploited to combat sea lice epizootics through selective breeding for resistant strains of the host salmon (Jones et al. 2002, Gjerde, Odegard & Thorland 2011). However, selective breeding for sea lice resistant

strains of salmon can be challenging (Glover, Nilsen & Skaala 2004) because factors affecting susceptibility of salmon to sea lice infection in the wild, farm and laboratory conditions are not completely understood (Glover et al. 2004, Hamre & Nilsen 2011). These factors can vary with strain of host and parasite, ecological circumstances, country/region, and farm management practices.

1.2.3.1. Host immunostimulation as a treatment strategy for sea lice infections

Innate immunity is the major means of protection against pathogen invasion in fish (Magnadóttir 2006, Whyte 2007) and its manipulation is being explored as a sea lice control strategy. Three main hallmarks of effective innate responses to sea lice are inflammation, cellular infiltration and hyperplasia. While chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon mount inflammation and hyperplastic responses at the sites of sea lice attachment and are able to reject the parasite shortly after infection (Johnson & Albright 1992, Braden et al. 2012); Atlantic salmon lacks such effective responses (Fast et al. 2003) despite induction of innate immune response genes (Skugor et al. 2008, Tadiso et al. 2011). This could explain the higher sea lice susceptibility of Atlantic salmon compared with coho, chinook and pink salmon (*O. gorbuscha*) (Johnson & Albright 1992, Jones, Kim & Bennett 2008, Wagner 2008), hence generating interest in inducing innate immune responses of Atlantic salmon as a control strategy against sea lice infection.

In the laboratory study by Sutherland et al. (2011), juvenile pink salmon weighing 0.3, 0.7 and 2.4 g were examined for transcriptional profiles 6 days post infection with *L. salmonis*. Results of the transcriptional analyses revealed susceptible, intermediate and resistant responses to sea lice infection for the 0.3, 0.7 and 2.4 g pink salmon groups, respectively.

Table 1.1. Summary of different strategies employed for the control of sea lice

Strategy	Scientific basis	Advantages	Disadvantages	References
Biological control	Natural predation on sea lice using cleaner fish such as wrasse	Cost effective, less environmental impact	Predator fish may transmit disease to salmon; also it may be invasive to the local fauna and flora; wrasse have also been shown to be more interested in eating the salmon feed rather than sea lice; and some species of wrasse do not over-winter well	(Deady, Varian & Fives 1995, Treasurer 2002, Groner et al. 2013)
Good management practices and monitoring	Maintenance of optimal environment for salmon health	Cost effective, less environmental impact	Has to be conducted in combination with other control strategies to maximize effectiveness	(Bron et al. 1993)
Selective breeding	Selection of sea lice resistant breeds of salmon	Low environmental impact	Takes a very long time to develop; may lead to unwanted traits such as poor food conversion ratio	(Jones et al. 2002, Gjerde, Odegard & Thorland 2011)
Immunostimulation	Boost the innate immunity of Atlantic salmon	Low environmental impact	Despite promising results (50% parasite reduction), no large scale field observation has been made so far	(Covello et al. 2011, 2012, Purcell et al. 2012, Poley et al. 2013)
Vaccines	Induction of acquired immunity of Atlantic salmon against sea lice	Low environmental impact	Not very effective; poor antibody response in Atlantic salmon probably due to lack of isolation of potent antigens within the sea louse	(Raynard et al. 1994, Grayson et al. 1995, Raynard et al. 2002, Ross et al. 2006, Frost, Nilsen & Hamre 2007, Carpio et al. 2011)
Drugs and chemicals	Selectively kill sea lice usually through neurotoxicity or disruption of molting cycle	Very effective especially shortly after introduction	Development of resistance to the agents over time leading to ineffective treatments; toxicity to non-target organisms and handlers; stressful to fish; expensive; may require withdrawal periods	(Burka et al. 1997, Burka, Fast & Revie 2012)

The higher susceptibility of the 0.3 g fish was linked to increased expression of matrix metalloproteinase (mmp)-9 and 13, together with cell proliferation inhibition-related genes (*sesn1*, *tob1* and *btg1*) culminating in cellular stress and possibly poor availability of nutrients to the fish at this stage of rapid development as a result parasite induced nutrient diversion. These changes were absent in the larger fish except for the 0.7 g fish which showed increased expression of mmp-9 and mmp-13, but not cell proliferation inhibition-related genes. Matrix metalloproteinase 9 plays a major role in inflammatory response to infection in fish (Chadzinska et al. 2008) and its up-regulation without simultaneous cell proliferation could have caused increased stress-induced susceptibility in the host. Several cell proliferation genes including protein phosphatase 1L, and Cdc37 [cell division cycle 37; chaperone involved in cell growth (Hunter & Poon 1997)] were up-regulated in the 2.4 g fish. Also, genes such as nucleolysin (*tia1*), involved in neutrophil activity and apoptosis of cytolytic lymphocytes (Tian et al. 1991), were up-regulated in the 0.7 and 2.4 g fish. Confirming the assertion by Johnson & Albright (1992) that neutrophils play a role in teleost defense against sea lice. Taken together, these findings indicate that manipulating the immune system of Atlantic salmon could result in reduced host susceptibility to sea lice infections. However, investigation into boosting Atlantic salmon innate immunity to sea lice yielded mixed results, depending on the type of immunostimulant used (Covello et al. 2012). Immunostimulants which have been used included β-glucans, occurring as polysaccharides in fungi, plants and bacteria (Tsoni & Brown 2008); cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN), unmethylated motifs found within bacterial and viral DNA (Carrington & Secombes 2006); and yeast extracts (Andrews et al. 2009). Covello et al. (2012) showed that whereas fish treated with β-glucan had higher sea lice numbers (24%) compared with untreated controls, CpG ODN and yeast extracts (AllBrew

NuPro) recorded 31-46% and 11-31% decrease in sea lice burden respectively. Cytosine-phosphate-guanine oligodeoxynucleotide, which resulted in the maximum percent reduction in sea lice infection, also caused mild to moderate inflammation and epidermal hyperplasia in the host, but not to the level seen in coho, pink and chinook salmon. The 3 immunostimulants used in the study by Covello et al. (2012) are highly conserved pathogen-associated molecular patterns (PAMPs) found in lower organisms, but absent in Metazoa (Tsoni & Brown 2008). Pathogen-associated molecular patterns are sensed by pattern recognition receptors (PRRs) such as Toll-like (TLR) (Bricknell & Dalmo 2005, Cuesta, Esteban & Meseguer 2008, Palti et al. 2010) and C-lectin (CLR) receptors (Tsoni & Brown 2008) in the host. On recognition of PAMPs, PRRs activate signaling pathways leading to transcription of genes involved in immune responses, including inflammation, antiviral responses and dendritic cell maturation (reviewed by Whyte 2007). Another study that investigated the effect of prior immunostimulation, using CpG and crude yeast extracts, on the efficacy of emamectin benzoate (EMB), revealed that Atlantic salmon that were treated with immunostimulants had lower numbers of sea lice compared with the control (Poley et al. 2013). Also, inclusion of CpG in the diet of Atlantic salmon re-infected with *L. salmonis*, resulted in lower sea lice numbers compared with first-time sea lice infections and the no treatment controls (Purcell et al. 2012). This observation, together with up-regulation of the inflammatory markers- mmp-9, IL-1β, IL-12β and down-regulation of TLR-9, suggest inflammatory and enhanced acquired immune responses in salmon against the sea lice for the CpG-treated fish. More studies on the exploitation of innate immune responses of Atlantic salmon as a treatment strategy against sea lice parasitosis are needed.

1.2.3.2. Use of drugs and chemicals in the control of sea lice

So far, the most effective and common means of sea lice control has been through the administration of chemicals or antiparasitic drugs either topically as bath treatments or orally in the feed. It is important to monitor sea lice abundance routinely (Heuch & Mo 2000, Treasurer & Pope 2000, Penston, Millar & Davies 2008) to determine timing for sea lice treatment, based on sea lice trigger levels (Heuch, Gettinby & Revie 2011), in order to delay development of drug resistance in the parasite population. This will be discussed further below. Also, the use of chemotherapeutants for sea lice control warrants monitoring for environmental impacts following treatment episodes (Davies et al. 2001, Willis & Ling 2003, Willis et al. 2005, Burridge et al. 2010). The major classes of drugs used for sea lice treatment (Table 1.2) are organophosphates (Bruno, Munro & McHenery 1990), pyrethroids (Hart et al. 1997), and macrocyclic lactones [MLs (Johnson & Margolis 1993, Stone et al. 2000b)]. Others include chitin synthesis inhibitors (Branson, Ronsberg & Ritchie 2000) and hydrogen peroxide (Johnson, Constible & Richard 1993, Bruno & Raynard 1994, Treasurer & Grant 1997, Bravo et al. 2010).

1.2.3.2.1. Advantages and disadvantages of different drug/chemical treatments

The advantages and disadvantages of the different types of sea lice treatment may vary depending on the type of chemical, dosage, and the mode of administration (Table 1.2).

1.2.3.2.1.1. Bath treatments

Bath treatment is the administration of drugs dissolved in water through complete immersion for a pre-determined period. One major advantage of bath treatments, especially when administered using well boats, is that all the parasites are exposed to the same concentration of the drug. This decreases the rate at which sub-therapeutic based drug

resistance/selection develops in sea lice compared with in-feed treatments. Until the mid-1990s, most bath treatments for sea lice control were performed with the organophosphates, metrifonate, dichlorvos and azamethiphos (O'Halloran & Hogans 1996). Later on, the pyrethroids (deltamethrin and cypermethrin) were introduced in several countries but not Canada; cypermethrin in 1996 and deltamethrin in 1998. Although pyrethrum was tested for sea lice control in Canada, its use and that of pyrethroids were not adopted for treating sea lice in Canadian salmon aquaculture (Roth 2000). However, recently, due to resistance development to EMB (the most widely used sea lice medicine) in Canada, deltamethrin (AlphaMax™) (Giffin et al. 2010) and azamethiphos were approved for use in New Brunswick.

Some of the bath treatment drugs had to be applied twice at 2 to 3 week intervals to achieve optimal efficacy as protection waned within a short time due to rapid reduction in therapeutic concentration of the drug within and around the sea-cage (Corner et al. 2011).

To achieve therapeutic concentrations during bath treatments, skirt enclosures, tarpaulins or well boats are used and extra supplies of oxygen provided. Food is also withheld. Procedures for bath treatments may be stressful for the fish, may cause minor injuries as fish are crowded and handled during treatment, and may reduce appetite post-treatment. When using H_2O_2, there is potential for high levels of re-infection due to resistance development (Treasurer, Wadsworth & Grant 2000). Therefore, farmers can potentially incur direct financial losses associated with the treatment, as well as with the potential for reduced growth of fish due to these secondary factors. Bath treatments are labor-intensive, and when using organophosphorous drugs, some salmon farmers may fail to follow recommendations to repeat the treatment, consequently encouraging the development of drug resistance by the parasite (Grave et al. 2004). If there are mobile stages of sea lice on the fish before or during the treatment, the farmer has to repeat the

treatment frequently (Mustafa, Rankaduwa & Campbell 2001). Apart from cypermethrin, bath treatments are only effective against preadult and adult stages of sea lice, allowing chalimus stages to survive and continue the cycle of infection. Treatments are, therefore, indicated only when populations reach preadult and adult stages and, thus, must be repeated frequently for effective control (Stone et al. 1999). Also the continual need for monitoring the efficacy of sea lice treatment results in further increases to production costs.

1.2.3.2.1.2. Pros and cons of in-feed treatments

In-feed treatments are administered to the fish with the drug directly milled into the diet. They are more advantageous compared with bath treatments because the former are less stressful to the fish, and relatively nonhazardous to the farmer (Ramstad et al. 2002). In-feed treatments allow medication during adverse weather conditions and permit simultaneous medication of all cages on a site and all sites in a loch, fjord, or single bay system, thus reducing cross-infection that commonly occur during the several days necessary to apply bath treatments to all cages on a site (Stone et al. 1999).

From 1996 to 2001, chitin-synthesis inhibitors were used as in-feed treatments, but their use was discontinued due to their lack of effect on the adult parasite following the final moult (Branson, Ronsberg & Ritchie 2000, Ritchie et al. 2002). For maximum efficacy and optimal sea lice control with chitin-synthesis inhibitors, treatments have to target developing stages of the parasite before the appearance of significant levels of the adult stage. Clearly, a treatment option that is efficacious against all parasitic stages of sea lice and which could be administered in feed, to avoid the disadvantages associated with bath applications and chitin-synthesis inhibitors, became highly desirable. The glutamate-gated chloride channels (GluCl) activators, avermectins (Smith & Clarke 1988, Stone et al. 1999), held such promise. However, due to the variability in

appetite among fish, and the natural hierarchies that exist within a cage, in-feed treatment has a greater probability of sub-therapeutic dosing of salmon than bath treatments. Also, potential variations in pharmacokinetics of individual fish will affect drug exposure to the attaching sea lice. Sick fish are likely to consume less feed/drug, leading to sub-optimal exposure of the parasite to the drug, consequently favouring development of resistance.

1.3. Avermectins

Avermectins are hydrophobic semi-synthetic MLs derived from a *Streptomyces avermitilis* culture discovered collaboratively by Merck & Co Inc, USA and the Kitasato Institute, Japan (Campbell 2012). Ivermectin (IVM) was the initial ML to be used in sea lice control (Smith & Clarke 1988, Johnson & Margolis 1993), but the high toxicity in the salmon host (Johnson & Margolis 1993, Davies & Rodger 2000) discouraged its continued use as a sea lice therapeutant. However, ivermectin was recently re-introduced as a sea lice medicine in New Brunswick. Another avermectin, doramectin, has been used for treatment of carp infected with the copepod anchor worm, *Lernaea cyprinacea* (Hemaprasanth et al. 2008), but not for sea lice. Ivermectin was shown to affect benthic organisms (Collier & Pinn 1998, Grant & Briggs 1998), hence the development of another ML, emamectin benzoate (EMB), which had a better therapeutic index (Roy et al. 2000) and is very effective for the control of sea lice parasitosis (Stone et al. 1999, Armstrong et al. 2000). Emamectin benzoate was initially developed and used as a pesticide against plant pests including lepidopterous insects (Leibee et al. 1995). The drug is marketed as a sea lice medicine under the brand name SLICE® and is administered in salmon feed at a dose of 50 μg kg^{-1} fish biomass for 7 days (Ramstad et al. 2002).

Table 1.2. Summary of different drugs and chemotherapeutants used in sea lice control

Drug	Class	Trade name	Dosage	Mechanism of action	Toxic dose (*Salmo salar*)	Thera-peutic margin	Target stage	References
				Bath treatment				
Dichlorvos	Organophosphate	Nuvan®, Aquagard®	1 mg L^{-1} per h	Inhibits AChE	> 4 mg L^{-1}	4x	Mobile stages	(Wootten, Smith & Needham 1982)
Trichlorfon	Organophosphate	Neguvon®	300 mg L^{-1} per h	Inhibits AChE	?	?	Mobile stages	(Costello 1993)
Azamethiphos	Organophosphate	Alfacron®/ Salmosan®	0.01 mg L^{-1} per h	Inhibits AChE	> 0.5 mg L^{-1}	5x	Mobile stages	(Roth & Richards 1992, Roth et al. 1996)
Carbaryl	Organophosphate	Sevin®	0.3-0.5 mg L^{-1} per h	Inhibits AChE	?	4- 7x	Mobile stages	(Costello 1993)
Pyrethrum	Pyrethrum	Py-Sal	10 µg L^{-1} - 10 mg L^{-1}	Sodium-gated chloride channel blocker	10 g L^{-1}, >2 min	4x	Mobile stages	(Costello 1993, Roth 2000)
Cypermethrin	Pyrethroid	Excis®/ Betamax®	5 µg L^{-1} per 30 sec	Sodium-gated chloride channel blocker	> 0.5 mg L^{-1}	100x	Mobile stages	(Hart et al. 1997)
Deltamethrin	Pyrethroid	AlphaMax®	3 µg L^{-1}	Sodium-gated chloride channel blocker	> 10 µg L^{-1}	0- 3.5x	Mobile stages	(Roth 2000)
Hydrogen peroxide	Oxidizer	Paramove®	1.5 mg L^{-1} per 20 min	Gas embolism	1.5-4g L^{-1}	0- 3x	Chalimus and mobile stages	(Treasurer & Grant 1997)
				In-feed treatment				
Teflubenzuron	Growth inhibitor	Calicide®	10 mg kg^{-1} per day for 7 days	Chitin synthesis inhibition	NA	NA	Larval stages	(Branson, Ronsberg & Ritchie 2000)
Diflubenzuron	Growth inhibitor	Lepsidon®	?	Chitin synthesis inhibition	NA	NA	Larval stages	(Roth 2000)
Ivermectin	Avermectin	Ivomec®	0.2 mg kg^{-1} 1x; 0.025 mg kg^{-1} 2x/week, 3 week; 0.07-0.08 mg kg^{-1} 1x/week, 3 week;	Glutamate-gated chloride channel activation	0.4 mg kg^{-1}, 1x; 0.05 mg kg^{-1} per 2 days, 2 weeks	2x	Chalimus and mobile stages	(Palmer et al. 1987, Johnson & Margolis 1993, Smith et al. 1993)
Emamectin benzoate	Avermectin	SLICE®	50 µg kg^{-1} per day for 7 days	Glutamate-gated chloride channel activation	0.36 mg kg^{-1}, 7 days	7x	Chalimus and mobile stages	(Roy et al. 2000)

AChE = acetycholinesterase; ? = unknown; NA = not applicable; mobile stages = preadults and adults

However, currently, double and triple doses are used in some salmon farms in Atlantic Canada (M. Beattie, Department of Agriculture, Aquaculture and Fisheries of New Brunswick, **http://www.dfo-mpo.gc.ca/science/enviro/aquaculture/rd2011/rdsealice-pou-eng.html**) as the manufacturer's recommended dose is no longer clinically effective. Emamectin benzoate, similar to other avermectins, is believed to act by blocking nerve transmission in arthropods resulting in starvation, paralysis and, consequently, death of the parasite (Arena et al. 1995, Stone et al. 1999). Unlike bath treatments, which are more labor intensive and can be stressful to the fish, EMB as an in-feed therapeutant is safely and effectively administered, and whole sites/bay systems can be medicated in a coordinated manner (Lees et al. 2008).

In a laboratory study to determine therapeutic dose and duration of administration for EMB in-feed treatment in Atlantic salmon experimentally infected with *L. salmonis*, the parasiticide had high efficacy against all parasitic stages of the parasite especially for the 50 μg kg^{-1} fish biomass for 7 days treatment group (Stone et al. 1999). The 50 μg kg^{-1} fish biomass treatment group recorded up to 95% parasite reduction which was higher than the 25 μg kg^{-1} fish biomass treatment group, but similar to the 100 μg kg^{-1} fish biomass treatment group. The multi-stage lethality of EMB disrupts the parasite life cycle at multiple points, thereby hampering propagation. In a field trial, in-feed treatment with EMB was shown to prevent development of copepodids for several weeks post-treatment compared with untreated control fish (Stone et al. 2000b). The efficacy of EMB was greater than 90% for at least 55 days from the start of treatment clearly showing that the parasiticide is not only highly effective in reducing existing sea lice burdens, but also prevents recruitment of new sea lice for several weeks from the initiation of treatment.

Sea lice collected during warmer temperatures appear to be more susceptible to EMB compared with those collected during the winter (Westcott et al. 2008) and environmental temperature has been described as a potential factor for EMB toxicity in *L. salmonis* (Lees et al. 2008). Results from experimental field trials with EMB-treated salmon revealed that treatment at higher temperatures (autumn) was more efficacious (63.3 - 99.3% efficacy) than during low temperatures (winter; 25.4 - 89.7% efficacy) (Stone et al. 2000b). However, the mechanisms of temperature effect on EMB efficacy require further investigation. Results to date suggest that seasonal/temperature-associated variation in EMB efficacy should be exploited in the timing of EMB treatment by administering the drug when efficacy has been shown to be optimal.

1.4. Pharmacology of emamectin benzoate as an ectoparasiticide for Atlantic salmon

1.4.1. Physicochemical properties of emamectin benzoate

The scientific name for EMB is (4″R)-5-O-demethyl-4″deoxy-4″(methylamino) avermectin A_{1a} and (4″R)-5-O-demethyl-25-de (1-methylpropyl)-4″-deoxy-4″(methylamino)-25-(1-methylethyl) avermectin A_{1a} (9:1) (Schering Plough Animal Health 1998). The benzoate salt of emamectin occurs as a white powder consisting of two components: 4'-epimethyamino-4'-deoxyavermectin B_{1a} benzoate (B_{1a}) and 4'-epimethyamino-4'-deoxyavermectin B_{1b} benzoate (B_{1b}). $B_{1a,}$ the active component, makes up ~90 % of the drug; the molecular formula is $C_{49}H_{75}NO_{13}C_7H_6O_2$ with molecular weight of 1008.26 g/mol. The molecular formula of the B_{1b} homologue is $C_{48}H_{73}NO_{13}C_7H_6O_2$ with a molecular weight of 994.24 g/mol. The two components differ from each other at the C26 position of the compound (Fig.1.2.). While B_{1a} has a methylene group on the isobutyl side chain, B_{1b} has a methyl group at that position. Ivermectin homologues differ at the C25 position (Shoop & Soll 2002); B_{1a} and B_{1b} possess a secondary butyl group and isopropyl moiety at this position, respectively. Similar to EMB, the B_{1a}

homologue of IVM makes up ~90 % of the drug. The solubility of EMB ranges from 24 (pH 7.04) to 320 (pH 5.03) mg L^{-1} in fresh water and ≤5.5 mg L^{-1} in salt water (Bright & Dionne 2005). Emamectin benzoate has an octanol-water coefficient (Log K_{ow}) of 5.0. The half-life of the drug is as follows: hydrolysis- ~136 days (pH 9, 25°C); photolysis- 1.4 to 22.4 days in solution; soil- 193.4, 427 and 174 days for aerobic, anaerobic, and at aerobic followed by anaerobic, respectively; and for marine sediment 164 to 175 days (Bright & Dionne 2005).

1.4.2. Pharmacokinetics of emamectin benzoate

The high lipid solubility of MLs plays a major role in their absorption in both host and parasites. Macrocyclic lactones accumulate in lipid tissue (Escribano et al. 2012) and are slowly released *in vivo,* hence causing sustained therapeutic concentration within the host (reviewed by McKellar & Gokbulut 2012, Geary & Moreno 2012). Lipophilicity of EMB is important in sea lice treatment because the drug persists in the fish at a therapeutic concentration for up to 9 weeks (at 13 - 15°C) following termination of administration (Sevatdal et al. 2005a), offering protection against sea lice for prolonged periods (Stone et al. 2000a).

When EMB is fed to fish, it is absorbed from the gut and redistributed throughout the fish with concentrations several folds higher in the skin compared with the muscle (Sevatdal et al. 2005a, Whyte et al. 2011). This is desirable for drug availability to sea lice through the mucus (Sevatdal et al. 2005a). Sea lice become exposed to the parasiticide primarily through ingestion of mucus of treated salmon, although cuticular absorption cannot be ruled out. This is because the attaching sea louse is submerged in salmon mucus while feeding on the host. By 63 days post-treatment, EMB concentration in the muscle returns close to insignificant levels (Skilbrei et al. 2008), an important consideration for determining the withdrawal period of the drug. A study by Sevatdal et al. (2005a) on the content of EMB in blood, mucus and muscle following field

administration of the recommended dose revealed that EMB concentrations reached maximum levels of 128, 105 and 68 ng/g (ppb) for blood, mucus and muscle respectively, on day 7, the last day of administration. From day 7 the concentration in the blood declined until it was less than the limit of detection on day 77. The concentration was higher in mucus compared with plasma, except on days 7 and 21. The concentration of EMB decreased gradually from the end of treatment (day 7) to day 70 with half-lives of 9.2, 10.0 and 11.3 days in muscle, plasma and mucus, respectively. The highest concentration of EMB in plasma was detected on the last day of treatment (day 7). For all other time points, the highest concentration of EMB was found in the mucus. Autoradiography revealed substantially higher EMB radioactivity in the intestinal mucosa than in other tissues for several days following administration. This could be linked to enterohepatic cycling of the drug as was shown for rainbow trout (*O. mykiss*) (Roy et al. 2006), and a consequence of very minimal metabolism of the parasiticide within the host (Kim-Kang et al. 2004). The parasiticide crossed the blood-brain-barrier (BBB), although quantities in the brain were lower than in muscle and blood for the first 4 days, but then exceeded those for the rest of the sampling period. How the kinetics of EMB and IVM differs at the BBB is discussed further below. The therapeutic effect of EMB may last less than six weeks in total, but with great inter-individual variation in protection, which may be a consequence of differences in appetite (Skilbrei et al. 2008) and the natural hierarchies that exist within a cage of Atlantic salmon.

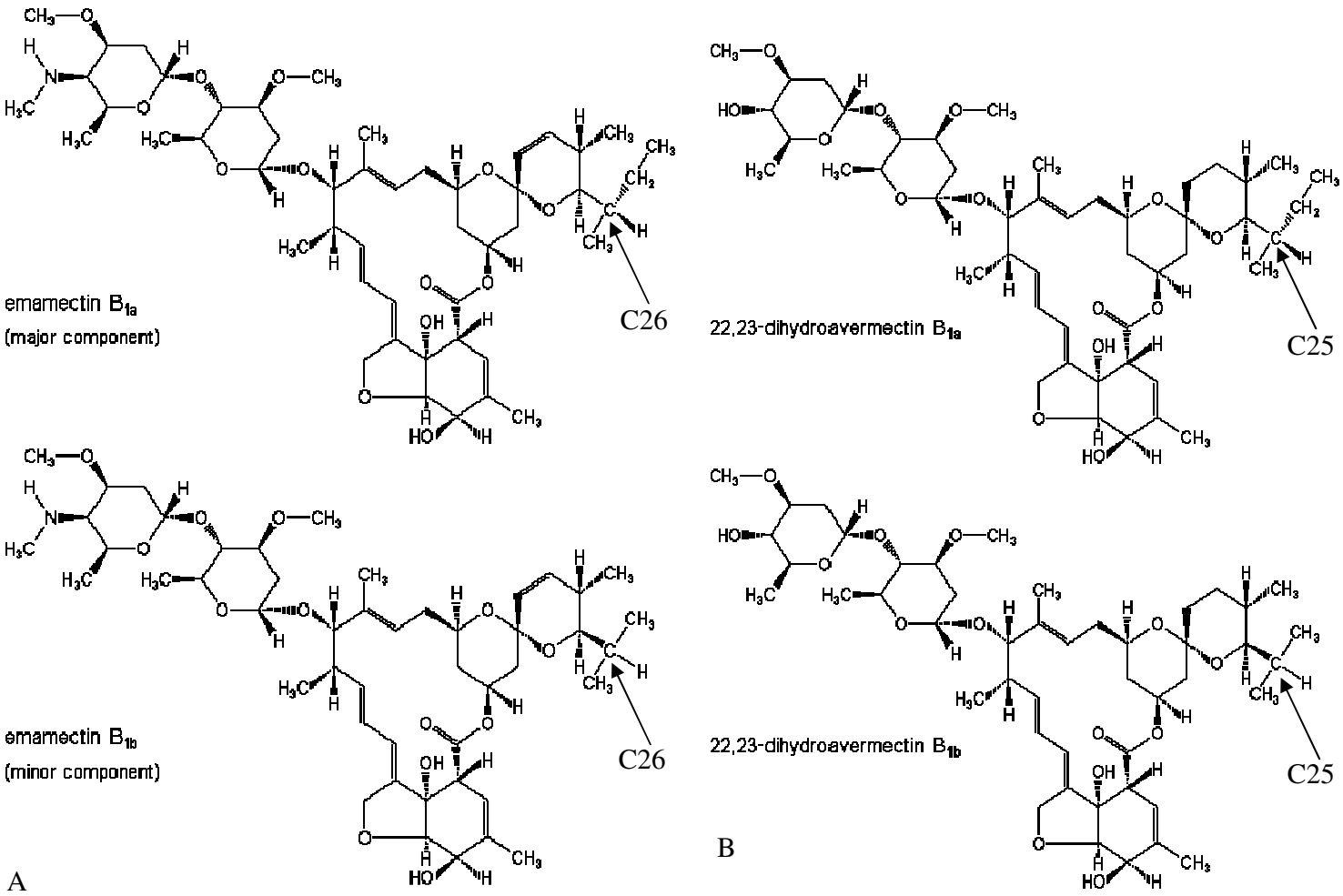

Figure 1.2. Chemical structures of emamectin [(A) (Bright & Dionne 2005)] and ivermectin [(B) (Shoop & Soll 2002)] homologues.

Temperature also plays a role in the pharmacokinetics of EMB in salmonids; as temperature increases, muscle half-life of the parasiticide decreases (Kim-Kang et al. 2004, Roy et al. 2006). This is an important consideration for the timing of EMB treatments between cold and warm seasons. For example, the mean water temperature in the Bay of Fundy in March and July of 2011 were 2.6 and 11.2°C, respectively (pers. comm. Dr. S.K. Whyte, Centre for Aquatic Health Sciences), and variations in seasonal temperature have been associated with seasonal differences in EMB efficacy in sea lice (Lees et al. 2008, Westcott et al. 2008).

1.4.3. Pharmacodynamics of emamectin benzoate

A great deal of what is known regarding the site and mechanism of action of EMB is extrapolated from studies on other MLs, particularly IVM, and mainly in nematodes. Hence, this section is based mainly on studies done in nematodes as well as the arthropod, *Drosophila melanogaster*. Most parasiticides exert their action through disruption of parasite neuromuscular systems (Geary et al. 1992, McVeigh et al. 2012). Macrocyclic lactones, including EMB, were initially presumed to act on γ-aminobutyric acid (GABA) receptors (Campbell et al. 1983), but the existence of glutamate-gated chloride channels (GluCls) in parasites was not known at the time (Geary & Moreno 2012). About a decade later, two GluCl subunits, α and β, were cloned from *Caenorhabditis elegans*, a nonparasitic nematode, and expressed in *Xenopus* oocytes (Cully et al. 1994). These subunits were capable of forming homomeric or heteromeric channels sensitive to glutamate, ibotenate, IVM and other avermectins. Glutamate-gated chloride channels are members of ligand-gated ion channels (LGICs) together with nicotinic acetylcholine, $GABA_A$, glycine (GlyR), serotonin 5-HT_3, cation channel selective *N*-methyl-D-aspartic acid (NMDA) and non-NMDA receptors (Cully et al. 1996). Ligand-gated ion channels are pentamers with subunits characterized by long N-terminal extracellular domain and four

membrane spanning domains with the second membrane-spanning domains forming the pore (Yates, Portillo & Wolstenholme 2003). Genes that encode LGICs are highly conserved in most nematodes, explaining why these channels are targets of many neurotoxic parasiticides (Williamson, Walsh & Wolstenholme 2007). Ligand-gated ion channel gene was also cloned from the mollusk, *Aplysia californica* (Kehoe et al. 2009). The GluClα subunit from *Haemonchus contortus*, a parasitic nematode, expressed in *Xenopus* oocytes, is activated by IVM and moxidectin in a slow but irreversible manner (Forrester et al. 2003). However, it is generally believed that avermectins act on both GABA and GluCl receptors (Yates, Portillo & Wolstenholme 2003), although GluCl seem to be the more widely accepted target site (Wolstenholme & Rogers 2005). Studies have shown that avermectins and milbemycins bind and activate *C. elegans* GluCl (Arena et al. 1995), suggesting that GluCl is the major target site of avermectin in nematodes (Yates, Portillo & Wolstenholme 2003). The high affinity binding of IVM at *H. contortus* GluClα (HcGluClα) is further proof that this receptor is an IVM target site in nematodes (Cheeseman et al. 2001). Other avermectins compete with IVM for the binding site on GluCl but their affinities are lower. Picrotoxin, fipronil, glutamate and GABA did not compete with IVM at this site, suggesting that they bind to different sites on GluCl channels (Yates, Portillo & Wolstenholme 2003). Ivermectin irreversibly activates *C. elegans* heteromeric GluCl α and β channels as well as GluClα homomeric channels in *D. melanogaster* (Cully et al. 1996), but studies in *H. contortus* and *C. elegans* showed that IVM does not bind to GluClβ subunit (Cheeseman et al. 2001), indicating selectivity for nematode GluClα subunit by the drug. Binding of IVM to the membranes derived from *C. elegans* was described as a two-step process (Schaeffer & Haines 1989) - an initial rapidly reversible binding characterized by a higher rate of dissociation followed by a slowly reversible binding which increases over time.

Binding affinity of the drug to *C. elegans* membranes was 100-fold higher compared with membranes from rat brain containing GABA receptors and was not affected by GABA, picrotoxin or bicuculline. Glutamate-gated chloride channel α and Rdl (resistance to dieldrin) subunits of GABA co-assemble as a heteromeric receptor for IVM in *D. melanogaster* head membranes (Ludmerer et al. 2002). Using anti-HcGluClα and anti-GABA antibodies, HcGluCl α and β subunits were localized in GABAnergic motor neurons of *H. contortus* (Portillo, Jagannathan & Wolstenholme 2003), further evidence that the two subunits form the receptor. *Haemonchus contortus* GluClα3A were localized with specific antibodies on amphidial structures, the major chemosensory organs in nematodes. The above-discussed studies, especially in nematodes, provide strong evidence that GluClα is the major target site for MLs.

Effects of IVM in *C. elegans* include inhibition of pharynx movement, motility and development of the larvae (Ardelli et al. 2009). Ivermectin and moxidectin caused reduced motility, paralysis and decreased fecundity in the filarioid parasite, *Brugia malayia* (Tompkins, Stitt & Ardelli 2010). Male *B. malayi* were more sensitive to the drugs compared with the females probably due to size differences; males are typically smaller in size than the females. This is contrary to observations made in sea lice, where female *L. salmonis* in laboratory bioassays were found to be more sensitive to EMB than the males (Westscott et al. 2008) and may be linked to intrinsically higher expression of P-glycoprotein (P-gp) mRNA in the male *L. salmonis* compared with the female *L. salmonis* (Chapter 2). Bioavailability of MLs in dogs and rats has been shown to be higher in females than males and was suggested to be due to differences in *P-gp* (protein) and/or other multidrug resistance (MDR) transporter activity or expression levels (reviewed by Lespine et al. 2009). The pharynx of the free-living nematode, *C. elegans*, was shown to be more sensitive to IVM than to moxidectin. Unlike in *C. elegans*,

electrophysiological recordings revealed that pharyngeal muscle of the chick luminal nematode, *Ascaridia galli*, is insensitive to IVM (Holden-Dye & Walker 2006), emphasizing the possible error associated with extrapolation of data from one species to another. Also, based on measurements of ingested ^{3}H-inulin, it seems that IVM has no effect on the feeding activity (i.e. no effect on the pharynx) of *H. contortus* (Sheriff et al. 2005), but on the contrary, IVM reduced the uptake of inulin in the parasitic nematode *Trichostrongylus colubriformis* (Sheriff et al. 2002), suggesting that the effects of this parasiticide may differ among different species of nematodes. An earlier study concluded that the major effect of IVM is paralysis of the pharynx in *H. contortus* (Geary et al. 1993). On the contrary, Sheriff et al. (2005) hypothesized that decreased motility rather than pharyngeal paralysis was the major effect of IVM in *H. contortus*. Since GluClα is expressed in the pharynx and somatic muscles of nematodes, these two effects may actually be occurring concurrently within these organisms following exposure to IVM (Wolstenholme & Rogers 2005). GluClα3B of *H. contortus* was shown to be more sensitive to glutamate and IVM compared with the corresponding subunit in *C. elegans* or the parasitic nematode *Dirofilaria immitis* (McCavera et al. 2009). Mutation at *D. melanogaster* GluClα caused decreased sensitivity to IVM, giving further evidence that the compound acts on this receptor (Kane et al. 2000). Higher concentration of IVM was needed to activate *C. elegans* GluCl receptors expressed in *Xenopus* oocytes compared with the concentration that paralyzes the pharynx and decreases worm motility of the free-living nematode *in vitro* (Yates, Portillo & Wolstenholme 2003). This was speculated to be due to differences in subunit composition between both systems or as a result of allosteric relationship that exists between the glutamate and IVM binding sites *in vivo* (Wolstenholme & Rogers 2005). Similar to its action on nematode GluCl channels, IVM acted as an agonist and irreversible activator of cloned human

GlyRs at low and high concentrations, respectively (Shan, Haddrill & Lynch 2001). It has also been suggested that IVM also acts through enhancing immune responses leading to rejection of microfilariae (*Brugia malayi*) in humans probably by inhibiting parasite secretions necessary for survival in the host (Moreno et al. 2010).

1.4.4. Toxic and side effects of emamectin benzoate in the host

The difference in therapeutic index between EMB and IVM in salmon has been linked to differences in *P-gp* substrate activity in the fish BBB (Horsberg 2012) and will be discussed further below. Although the two avermectins were shown to cross the salmon BBB (Høy, Horsberg & Nafstad 1990, Sevatdal et al. 2005a), IVM accumulated in salmon brain at toxic levels (Høy, Horsberg & Nafstad 1990), whereas EMB is likely pumped directly out on crossing the BBB explaining its relatively lower concentration in the teleost brain (Sevatdal et al. 2005a). Concentration of IVM was higher in the brain compared with the muscle at 2 days post-administration and all through the sampling period (28 days), except at 12 and 24 h post-administration (Høy, Horsberg & Nafstad 1990). However, EMB attained higher concentrations in the brain compared with the muscle at 28 days post-administration (Sevatdal et al. 2005a). Also, while EMB caused toxicity (lethargy, dark colouration, depressed appetite and poor coordination) there was no mortality in salmon at ~7x the recommended dose (356 µg kg^{-1} fish biomass day^{-1} for 7 days) (Roy et al. 2000), whereas oral administration of 400 µg kg^{-1} fish biomass IVM as a single dose to Atlantic salmon resulted in higher mortality compared with 200 µg kg^{-1} fish biomass treatment (Palmer et al. 1987). These studies suggest differences between the affinity of EMB and IVM to *P-gp* at the salmon BBB. The presence of *P-gp* in vertebrate (mice) BBB causes the efflux of IVM away from the brain (central nervous system) (Schinkel et al. 1994) limiting access of the drug to GABA receptors located in the brain (Kiki-Mvouaka et

al. 2010). Using wild-type and *mdr1ab* (−/−) knockout mice Kiki-Mvouaka et al. (2010) showed that the transporter affected absorption of IVM and eprinomectin and also caused increased elimination of eprinomectin, but had insignificant effects on the kinetics of moxidectin. These findings suggest that the effect of *P-gp* on toxicity of MLs may vary. There are also species differences in the role of *P-gp* in the toxicity of MLs. Mutation in the *mdr1* gene (which codes for *P-gp*) that gives rise to a sudden stop to P-gp mRNA translation was linked to increased IVM sensitivity in collies (Mealey et al. 2001). The mutation was shown to be either a 4-bp GATA or ATAG deletion (Roulet et al. 2003). Disruption of this gene caused increased IVM toxicity in mice (Schinkel et al. 1994), most likely due to compromised BBB confirming the role of *P-gp* in IVM distribution (Mealey 2008).

In a laboratory study to determine the margin of safety of EMB in Atlantic salmon, *S. salar* and rainbow trout, *O. mykiss*, the parasiticide was well tolerated by both salmonids for up to 3-4 times the recommended dose (50 μg kg^{-1} fish biomass for 7 days) (Olsvik et al. 2008). However, analyses of hepatic genes in Atlantic salmon following 50 μg kg^{-1} EMB treatment revealed moderate (mostly < 2-fold) effects. These included genes encoding proteins involved in modification processes (such as acetylation, methylation or phosphorylation), inflammatory responses, and protein binding and nucleotide cleavage (Olsvik et al. 2008). This shows that although EMB is administered to kill sea lice, the effect of the drug on host salmon may be present at the molecular level, especially on the stress and inflammatory processes, possibly interfering with the host-parasite relationship. This should be considered particularly in salmon farms locations, for example in the Bay of Fundy, New Brunswick, Canada, where currently 2-3x the recommended dosage are administered to the fish due to lack of clinical effectiveness of the manufacturer recommended dose within some areas in the Bay (M. Beattie, Department of

Agriculture, Aquaculture and Fisheries of New Brunswick, **http://www.dfo-mpo.gc.ca/science/enviro/aquaculture/rd2011/rdsealice-pou-eng.html**).

1.4.5. Fate and effects of emamectin benzoate on the environment

Similar to most parasiticides, drugs and chemicals used for sea lice control, including EMB, are potentially toxic to non-target and benthic organisms (Burridge et al. 2010) especially crustaceans (Willis & Ling 2003, Waddy et al. 2007). This is because the action of these parasiticides on sea lice is non-specific. Hence the drug/chemical will affect most other organisms that are phylogenetically close to sea lice and/or possess the target sites for the parasiticide. Accumulation of EMB in sediments around sea cages arises from uneaten medicated feed as well as from fish faecal matter containing the parent compound and its desmethylamino metabolite. Emamectin benzoate undergoes very minimal metabolism within the host (Kim-Kang et al. 2004); hence the fish faeces will contain more of the parent drug than its metabolite. Grave et al. (2004) showed that the rate of sea lice treatments, including EMB, in Norway, increased over time (from 1988 to 2002) due to reduced efficacy and/or increase in fish biomass. This implies that higher concentrations of sea lice medicine are introduced into the environment over time. Since EMB has low water solubility and high Log K_{ow}, there exists the potential for the drug to persist in the marine environment tightly bound to sediments for prolonged periods (Bright & Dionne 2005, Burridge et al. 2010). An investigation into the environmental effects of EMB in a commercial salmon farm (Telfer et al. 2006) concluded that the maximum concentration of the drug in the sediments (2.73 µg kg^{-1} wet weight) had no toxic effects on free-living organisms such as crabs, polychaetes and whelks within 10 m of the sea cages. Similar to EMB, IVM has the potential to accumulate in the sediment, and may cause mortality among polychaetes over time (Black et al. 1997, Collier & Pinn 1998). Although, an

earlier study reported that in-feed IVM treatment at 50 µg kg^{-1} twice per week for 3 months did not affect polychaetes resident in sediment under the treated sea cages (Costelloe et al. 1998). Emamectin benzoate was shown to cause premature molting in American lobsters (*Homarus americanus*) (Waddy et al. 2007), but that this occurs if a 500-g lobster consumes a minimum of 110 µg of EMB (22 g of medicated feed) which is highly unlikely. The effect of EMB on benthic organisms varies widely. A 48 h acute toxicity test (carried out in the laboratory with filtered sea water) using marine copepods (*Acartia clausi*, *Pseudocalanus elongatus*, *Temora longicornis* and *Oithona similis*) revealed 5 to 2000-fold higher EMB EC_{50} for *O. similis* compared with the other copepods (Willis & Ling 2003). Also, the blue mussels (*Mytilus edulis*) can bioaccumulate IVM (up to 5.2 mg kg^{-1} mussel tissue) when exposed to very high concentration 6.9 µg L^{-1} of the parasiticide in the laboratory for 6 days and it has a depuration half-life of 22 days (Davies, McHenery & Rae 1997). Although degradation of IVM in the environment is slow and the half-life may be more than 100 days (Davies et al. 1998), such high concentrations of IVM (6.9 µg L^{-1}) are not normally obtained in the environment (Davies, McHenery & Rae 1997). However, prolonged use of higher concentrations of avermectin sea lice medicines such as EMB (as reported in some salmon farms in Atlantic Canada), for example due to resistance development, may lead to accumulation and persistence of high levels of the drugs in the sediments. There is the need to exercise caution in using higher concentrations of EMB or frequent repeat treatments for sea lice control, as the drug has the potential to persist in the sediment and affect non-target benthos within the exposed site.

1.5. Parasiticide resistance

Resistance is the heritable loss of sensitivity to a drug by target organisms (Sangster 2001, He et al. 2009) and usually manifests when most individuals within the population survive

doses of the drug that kill a 'normal' population of the same species (Prichard et al. 1980). Loss of drug efficacy is described as cross-resistance when the affected drugs possess different modes of action (Prichard et al. 1980). Multiple-resistance occurs when a parasite population becomes insensitive to several drugs with different mechanisms of action (Wrigley et al. 2006).

The effectiveness of EMB and its advantages over other chemotherapeutants quickly made it the drug of choice with almost exclusive use for sea lice control in salmon farms in the Bay of Fundy, NB (Westcott, Hammell & Burka 2004) and elsewhere. However, the use of EMB has sharply declined in recent years due to resistance development (discussed below). Widespread drug resistance to available sea lice therapeutants, especially to EMB, has exacerbated the challenge of sea lice control.

1.5.1. Sea lice resistance to emamectin benzoate

Resistance has developed to various parasiticides used to control salmon lice (Denholm et al. 2002, Grave et al. 2004), numerous parasites of land-based animal husbandry around the world (Le Jambre 1993, Swan et al. 1994, West et al. 1994, Vermunt, West & Pomroy 1996, Jackson et al. 2006, Waghorn et al. 2006, Slocombe, de Gannes & Lake 2007, Howell et al. 2008, Sutherland et al. 2008, Condi, Soutello & Amarante 2009, Demeler et al. 2009, Gasbarre et al. 2009, Edmonds, Johnson & Edmonds 2010, Perez-Cogollo et al. 2010), as well as the parasitic nematode *Onchocerca volvulus* in human filariasis (Osei-Atweneboana et al. 2007). Hence, reliance on drugs and chemicals can only serve as short-term solutions for the control and management of most parasitic organisms (Prichard et al. 1980).

The limited range of treatment options and control strategies for several years has contributed to resistance development in sea lice (Denholm et al. 2002). Although the need for chemical diversity and the avoidance of over-dependence on a single chemotherapeutant cannot

be over-emphasized (Sangster 2003, Wolstenholme et al. 2004), the tendency to rely on single products for salmon lice control still exists. This may be traced to the difficulty in developing and licensing new drugs (Denholm et al. 2002) and the reliance of farmers on available therapies that maintain their efficacy. In Canada, regulatory bottlenecks are some of the major challenges towards developing or approving new drugs for sea lice control. Emamectin benzoate was initially approved for use in Canadian salmon aquaculture in 1999 by Health Canada Veterinary Drugs Directorate under the Emergency Drug Release programme, but it did not receive full drug approval until 2009.

For several years, EMB resistance development was a major concern for *L. salmonis* control due to over-reliance on the parasiticide by salmon farmers (Westcott, Hammell & Burka 2004). Consequently resistance development to the drug by *L. salmonis* has been reported in Atlantic Canada and Europe (Hjelmervik et al. 2010, Westcott et al. 2010) and by *Caligus rogercresseyi* in Chile (Bravo, Sevatdal & Horsberg 2008, Horsberg 2012). This emphasizes the need for early detection of changes in EMB sensitivity as part of resistance management strategies (Westcott et al. 2008). A study to determine temporal changes in EMB efficacy in different sea lice life stages using data collected from salmon farms in New Brunswick between 2004 and 2008 revealed a decrease in EMB treatment efficacy (Jones et al. 2013). The study also showed that EMB sensitivity varied among different farm locations and decreased at a faster rate in NB compared with analysis of data collected from salmon farms in Scotland between 2002 and 2006 (Lees et al. 2008).

In a study by Bravo et al. (2008), loss of sensitivity in *C. rogercresseyi* to EMB was attributed to the exclusive use of EMB to control sea lice in Chile for more than seven years, coupled with the previous employment of IVM, used for approximately ten years during the

1990s. This favoured selection for resistance towards this class of parasiticides. The sensitivity studies using bioassays were performed between 2006 and 2007 when clinical failures of EMB treatments were already evident, making it difficult to determine EMB sensitivity in naïve parasites for comparison purposes; although the authors suggest a loss of sensitivity in comparison with *L. mugiloidis* control (Bravo, Sevatdal & Horsberg 2008). Since EMB has been used extensively in doses higher than the recommended dose and for extended treatment periods without yielding tangible clinical effectiveness, it is probable that EMB eliminated susceptible parasites leading to the propagation of EMB resistant strains of sea lice (Bravo, Sevatdal & Horsberg 2008). In contrast to the study by Bravo, Sevatdal & Horsberg (2008), EMB bioassay studies carried out with *L. salmonis* collected from the Bay of Fundy, NB from 2002 to 2005 prior to resistance development (Westcott et al. 2008) showed that the parasite were relatively more EMB sensitive in the latter study. In addition to the prior use of IVM in Chilean salmon aquaculture, the differences in EMB sensitivity between the studies by Bravo et al. (2008) and Westcott et al. (2008) may be linked to duration of use of the parasiticide. While EMB was approved as the only sea lice medicine in Chilean salmon aquaculture in 2000, the drug gained full approval for use in Canada only in 2009.

The high efficacy and broad spectrum of activity of MLs led to over-dependence on this class of drugs for parasite control in plants, animals and man, globally. The over-dependence has consequently led to emergence of resistant strains of the target parasites to the different MLs over time. *H. contortus*, a common trichostrongylid parasite of ruminants, developed resistance to MLs, including IVM (Blackhall et al. 1998a). Ivermectin, the only available drug for mass treatment of onchocerciasis (caused by *O. volvulus*) and lymphatic filariasis in West Africa and the Americas, selected for resistant parasites over time, due to therapeutic pressure (drug

selection of resistant strains of the parasite), leading to treatment failure (Eng et al. 2006). Hence, there is widespread resistance development to different MLs as well as the other major classes of parasiticides, and an urgent need for the development of new classes of parasiticides as well alternative control strategies.

Work to understand development of drug resistance in *L. salmonis* is of practical importance (Denholm et al. 2002) because such knowledge is necessary for implementing effective drug rotations. It is widely believed and commonly observed that drugs in the same class with similar mechanism of action select for the same resistance mechanism, especially for resistance development involving target site mutation or increased/decreased metabolism.

1.5.2. Resistance mechanisms

Understanding drug resistance mechanisms, especially at the molecular and/or receptor level, is necessary for tracking resistance development (Blackhall et al. 1998a, Sangster & Gill 1999, Prichard et al. 2007) and for responsible use of different classes of parasiticides (Geary, Sangster & Thompson 1999). Two important concepts usually considered in drug resistance are: 1) drug binding at the target site and 2) therapeutic concentration of the drug at the target site. These two concepts, usually associated with the different resistance mechanisms, are discussed below.

1.5.2.1. Target site

Resistance at the target site is generally due to mutations that affect the ability of the drug to bind to its target, as shown for the organophosphate binding site on acetylcholinesterase (AChE) in sea lice (Jones, Sommerville & Wootten 1992, Denholm et al. 2002, Fallang et al. 2004). Also, drug resistance can occur due to down-regulation of gene expression leading to a

reduction in the expression of the corresponding target protein as has been shown for avermectins in *H. contortus* (Blackhall et al. 1998b, Wolstenholme et al. 2004). Mutation at the Dα6 subunit of nicotinic acetylcholine receptor (nAChR) was linked to specific and high-level spinosyn (a tetracyclic-macrolide) resistance in *D. melanogaster* (Watson et al. 2010), but no cross-resistance was observed to avermectins, pyrethroids, oxadiazines, neonicotinoids and nicotinics. Silencing of the Dα6 nAChR subunit gene conferred high-level spinosyn resistance in *D. melanogaster* (Perry, McKenzie & Batterham 2007).

Pyrethroid resistance, an example of knockdown resistance (kdr), which arises from point mutations in the target site, the para-type sodium channel of nerve membranes (Fallang et al. 2005) has been reported for deltamethrin in sea lice (Sevatdal & Horsberg 2003, Fallang et al. 2005). The two pyrethroids with approval for salmon lice control (deltamethrin and cypermethrin) are structurally similar and are expected to be cross-resisted through target-site mutation (Denholm et al. 2002).

Macrocyclic lactone resistance has been linked to changes in the target site and an increase in drug efflux (reviewed by Wolstenholme, Kaplan 2012). Most of the investigations into the involvement of target site changes in ML resistance utilized nematodes. Mutations in GluCls have been implicated in resistance to IVM in both parasitic and free-living nematodes (Dent et al. 2000, Njue et al. 2004, McCavera et al. 2009) and may be polygenic, requiring mutation in different GluCl genes before high level resistance occurs. Simultaneous mutation of the GluCl genes, *avr-14*, *avr-15*, and *glc-1*, conferred over 4000-fold resistance to IVM in *C. elegans* (Dent et al. 2000). Ivermectin and moxidectin selected one allele of GluClα subunit, but none at the β subunit, suggesting that resistance to the avermectins is due to target site mutation at the α subunit (Blackhall et al. 1998a). Such target site mutations can also be useful in tracking

resistance development to this family of parasiticides. Mutation in GluClα3 and GluClβ subunits located at the N-terminal extracellular domain caused an approximate 2.5-fold decrease in IVM and moxidectin sensitivities in the parasitic nematode *Cooperia oncophora* (Njue et al. 2004). Differences in allele frequency of GluClα3 were reported between IVM - resistant and - sensitive *C. oncophora*, whereby alleles E and H were higher in the resistant strain than in the sensitive strain, while allele A was higher in the sensitive strain than in the resistant strain (Njue & Prichard 2004). Also, some studies have linked ML resistance to mutation at GABA receptors, suggesting these receptors are targets for ML activity. Ivermectin and moxidectin selected for resistant alleles for genes encoding *H. contortus* GABA receptors (Blackhall, Prichard & Beech 2003). Ivermectin susceptible and resistance subunits of GABA-A receptor α/γ, *HG1A* and *HG1E*, respectively, were cloned from *H. contortus* (Feng et al. 2002). However, results from binding studies using membranes from IVM-sensitive and -resistant *H. contortus* L_3 larva suggest that target site change is not involved in IVM resistance (Rohrer et al. 1994). Also, comparison of cDNA sequences of IVM-sensitive and -resistant *H. contortus* revealed no coding differences, suggesting that either IVM does not select for an existing target site mutation in the parasite or that the putative mutation occurs in the non-coding region of the GluCl gene (Cheeseman et al. 2001). Ivermectin resistance was linked to GluClα mutation in *D. melanogaster* (Kane et al. 2000). Also, selection for abamectin resistance in the two-spotted spider mite, *Tetranychus urticae*, was reported to cause resistance to milbemectin (Sato et al. 2005). This is an important consideration for sea lice control using avermectins. For example in the Bay of Fundy, although most salmon cage sites are already reporting reduced EMB efficacy, IVM has been re-introduced for the control of sea lice.

In addition to the issue of resistance among MLs, cases of cross-resistance have been described for this class of drugs whereby the use of a particular ML selected for resistance to another drug belonging to an entirely different class. A classical example of this phenomenon is the study where selection for avermectin-resistant strains of *H. contortus* using IVM and moxidectin treatment alone also selected for benzimidazole resistance (de Lourdes Mottier, Prichard 2008). Avermectin-induced benzimidazole resistance was linked to selection for single nucleotide polymorphisms (SNPs) from TTC (Phe) to TAC (Tyr) at codon 200 and 167 in the β-tubulin isotype-1, but not isotype-2 or α-tubulin. However, benzimidazole resistance did not confer IVM resistance in nematodes (Eng et al. 2006). Restriction fragment length polymorphism and single strand conformational polymorphism analyses of several genes in *O. volvulus* isolated from humans treated or not treated with IVM revealed genetic polymorphism in β-tubulin (Bourguinat et al. 2007, Lustigman & McCarter 2007), ATP-binding cassette (ABC) transporter homologue OvABC-3 (Ardelli & Prichard 2007) and P-gp genes (Eng & Prichard 2005) (to be discussed below). Ivermectin and moxidectin selected for different alleles of *H. contortus* P-gp gene compared with untreated parasites (Blackhall et al. 1998b).

A change in amphidial structure has also been linked to IVM resistance in *H. contortus* (Freeman et al. 2003), as noted earlier. The study by Freeman et al. (2003) showed that dendritic processes of IVM-resistant strains of *H. contortus* were further away from amphidial pore openings compared with the same structures in sensitive strains of the parasite.

1.5.2.2. Decreased drug uptake

P-glycoprotein, a member of ABC transporters that belong to integral plasma membrane proteins, causes the efflux of a diverse range of molecules from within cells to the exterior (Fojo et al. 1985, Raviv et al. 1990). Although genetic changes in GluCl channels have been

associated with ML resistance (Njue et al. 2004), increased expression of *P-gp* is widely believed to be the primary mechanism responsible for loss of parasite sensitivity to MLs (reviewed by Prichard & Roulet, 2007). P-glycoprotein appears to play a major role in the protection of aquatic invertebrates from xenobiotics (Smital & Kurelec 1998, Fulton et al. 1999, Lyons-Alcantara et al. 2002).

Macrocylic lactones are substrates for *P-gp* and have high binding affinity for the transporter (Lespine et al. 2007). Increased expression of *P-gp* was linked to ML resistance in *C. elegans* and *H. contortus* (Blackhall et al. 1998b, Xu et al. 1998, Sangster et al. 1999, James & Davey 2009). Using *in situ* hybridization, P-gp mRNA was localized in the digestive tract and pharynx of *H. contortus* (Smith & Prichard 2002). However, the level of expression in the pharynx did not differ between IVM and moxidectin resistant and sensitive strains of the parasite. Some studies have shown that acute and chronic inflammatory responses can increase or decrease P-gp expression, respectively (Dumoulin et al. 1997, Ho & Piquette-Miller 2006). Therefore, although inflammation plays a positive role in the rejection of sea lice in salmonids (Johnson & Albright 1992, Jones, Kim & Bennett 2008, Wagner 2008), concurrent administration of EMB and immunostimulants to Atlantic salmon may, in fact, favour resistance development to the drug (Chapter 3).

Macrocyclic lactone resistance can be reversed using modulators of the pump, such as verapamil (Molento & Prichard 2001), confirming the role of ABC transporters in IVM resistance. Reversal of ABC transporters is a potential means of enhancing ML efficacy (Bartley et al. 2009), but differences in the kinetics of different MLs and existing reversal agents and potential toxicity of the latter may hamper realistic use of this strategy (Lespine et al. 2008).

Apart from being substrates of *P-gp*, MLs have also been shown to reverse *P-gp*-associated multidrug resistance in mammalian cells (Pouliot et al. 1997, Griffin et al. 2005).

Based on several studies associating *P-gp* with resistance to IVM in parasitic and free-living invertebrates, it was logical to investigate the involvement of this transporter in EMB resistance in sea lice. It is hypothesized that up-regulation or over-expression of the P-gp gene in the gastrointestinal epithelium would serve to limit the absorption of various compounds, including EMB, ingested by the salmon louse (Tribble, Burka & Kibenge 2007). A putative *L. salmonis* P-gp was cloned based on sequences of the transporter in the GenBank database at the time (Tribble et al. 2008). However, with the cloning and addition of more P-gp sequences to the GenBank database, the putative ABC transporter reported by Tribble et al. (2008) was discovered to be a mitochondria half-transporter (Heumann et al. 2012). A novel *L. salmonis* P-gp SL-PGY1 (GenBank accession number HQ684737) was cloned by Heumann et al. (2012). Nematodes have numerous P-gp genes compared with mammals with *C. elegans* possessing up to 15 (Prichard & Roulet 2007); the number of P-gp genes in sea lice is unknown. A component of the current study investigating the relative expression of the P-gp gene in *L. salmonis* is based on the SL-PGY1 sequence reported by Heumann et al. (2012).

1.5.2.3. Reduced metabolism

Resistance can also result from enhancement of detoxification systems, for example through amplified esterases (Denholm et al. 2002) such as cytochrome P450 monooxygenase in pyrethroid-resistant sea lice (Sevatdal et al. 2005b). It is not known whether EMB undergoes any significant enzymatic breakdown within the salmon louse. Emamectin benzoate undergoes limited metabolism in Atlantic salmon (Kim-Kang et al. 2004) and, if this can be extrapolated to the parasite, would suggest that changes in metabolism would not significantly contribute to

EMB resistance in the salmon louse. Also, EMB is a lipophilic drug, hence is slowly redistributed and metabolized in the salmon. Therefore, any changes in metabolism should not significantly affect concentration of the drug within the parasite.

1.5.3. Environmental and genetic factors affecting parasiticide resistance

Drug resistance is an evolutionary adaptive process where resistant strains of a parasite are favoured through drug selection pressure to become the predominant strain in the population (Sangster 1999). Concurrent disease outbreaks including high sea lice infections can also cause depressed appetite (Damsgård, Mortensen & Sommer 1998) in affected fish leading to poor uptake of EMB during treatment (Berg & Horsberg 2009). Such situations increase the rate at which resistance to the drug develops, i.e. consequence of sub-therapeutic EMB concentrations in the mucus of sick fish. Also, affected fish can possibly serve as a refuge for drug-selected sea lice within the cage and, thus, a source of re-infection for healthy fish, further driving drug selection for resistant strains of the parasite.

The presence of *L. salmonis* originating from a large wild salmon population in Pacific Canada is a likely source of EMB-sensitive sea lice to the salmon farms in the region, which should dilute any rise in EMB-resistant sea lice populations (Saksida et al. 2012). Also, threespine stickleback (*Gasterosteus aculeatus*) has been reported as a host for sea lice in Pacific Canada (Jones & Prosperi-Porta 2011). In contrast, wild salmon populations in the Atlantic have declined considerably (Anderson, Whoriskey & Goode 2000) and not a significant source of sea lice. Also, the threespine sticklebacks are not reported to be infected with *L. salmonis* in this region. These indicate decreasing possibilities of non-farm "wild" sea lice diluting a rising EMB-resistant sea lice population in the Atlantic region. This may explain the higher incidence of EMB resistance in salmon farms located in Atlantic Canada compared with the Pacific (Jones

et al. 2012). It is not known whether sea lice have any other wild host species in Atlantic Canada.

Resistance is a consequence of changes in the genetic profile of the parasite population resulting in a phenotype of reduced sensitivity to treatment (Eng & Prichard 2005). Genes conferring resistance arise through mutation, but maintain very low frequencies in pest populations in the absence of drug exposure (Denholm et al. 2002). Over time and with recurrent treatments, genes conferring resistance are passed from one generation of survivors to another (Sangster 1996, Sangster & Gill 1999). Following each exposure, individuals possessing these genes are selectively favoured and increase in frequency within a population. Early on, the number of resistant survivors may be too low to affect treatment outcome, but over time and, with continued drug selection, the survivors become the dominant individuals within the population causing severe treatment failures (Denholm et al. 2002). More genetically variable organisms possess greater capability of having an allele capable of causing poor treatment outcomes. The evidence for the involvement of a gene in resistance to a drug can be obtained by examining the genetic variability of the gene between individuals sensitive and resistant to the drug (Blackhall et al. 1998a), where available. The speed at which resistance develops and its extent depends on the virulence of the parasite, type of resistance mechanism, the frequency and level/dose of chemical use, and the parasite biology (Denholm et al. 2002). Variations in resistance to sea lice therapeutants could be due to phenotypic or genetic differences and has been examined using different approaches (reviewed by Boxaspen 2006). Provided the appropriate genes are investigated, changes in genetic profile are likely to be evident prior to widespread resistance-induced treatment failure and such changes in genetic disposition could be used to monitor the development of resistance to the parasiticide in use (Eng & Prichard 2005).

The application of population genetics promises to be a useful tool in the analysis of drug resistance in parasites and other pest species (Blackhall et al. 1998a). Mutations and/or over-expression observed in drug resistant parasites, but not in sensitive strains, can be expected not only in genes involved in resistance mechanisms, but may also be seen in genes upstream of the resistance activation cascade such as the nuclear receptor genes. Identifying genes associated with drug resistance selection will be useful in the development of possible markers for tracking reduced drug sensitivity as it develops (Sangster et al. 2002). Control programs that combine genetic, parasitological, and clinical aspects of treatment are usually more effective than any one aspect of monitoring (Eng & Prichard 2005).

1.5.4. Diagnosis of drug resistance

Detection of anthelmintic resistance in nematodes of terrestrial livestock is mainly based on *in vivo* (e.g. fecal egg count reduction) and *in vitro* (e.g. egg hatch/larval development) tests (Coles et al. 1992), which have been employed in several studies to monitor ML efficacy (Coles et al. 2006, Yazwinski et al. 2009). Monitoring is important to the rotational use of parasiticides so as to detect changes in sensitivity and delay resistance development (Zhao et al. 2006). Similar to the key factors discussed in the review by Zhao et al. (2006) on monitoring anthelmintic resistance in human onchocerciasis, including host, stage of parasite, and timing of monitoring (Churcher & Basanez 2009), proper monitoring of all stages of sea lice is important for the strategic timing of treatments of farmed salmon (Brooks 2009). Depending on locality/region, reduced sensitivity and potential resistance to currently available medicines are constant threats to the control of sea lice populations on salmon farms. Hence there is the need for on going monitoring of treatment efficacy.

Bioassays are commonly used to measure clinical effectiveness and investigate the effects of EMB on gene expression in surviving sea lice (Tribble, Burka & Kibenge 2007). Although the bioassay protocol shows promise as a method to verify clinical resistance, it lacks rapidity and simplicity for use as a routine test (Westcott et al. 2008). Thus, initial work to develop bioassay methods has focused on testing preadult and adult sea lice that have been removed from their hosts. This has several implications. Firstly, once removed, the sea lice can only be used within a limited time frame, usually within 48 h, for meaningful results. Secondly, endpoints can be hard to define, as the parasites can survive in a moribund stage for prolonged periods following exposure to most control agents (Denholm et al. 2002). Hence bioassays are best suited for measuring indicators of sensitivity to the parasiticide under study by using a range of doses which include those that have been shown to be effective (Westcott et al. 2008). Monitoring strategies should be precise and practicable. They should also be robust, simple and repeatable with an unambiguous endpoint as well as having sufficient sensitivity to detect changes in efficacy of the parasiticide. However, no single resistance bioassay is likely to deal adequately with all chemicals in use, due to differences in their physico-chemical properties, life-stage specificity and speed of action (Denholm et al. 2002).

Assuming a relationship between drug resistance and its biochemical or molecular basis can be established, *in vitro* diagnostics for specific resistance mechanisms offer the prospect of more rapid tools for resistance monitoring (Denholm et al. 2002, von Samson-Himmelstjerna & Blackhall 2005). The narrow spectra of available treatment options and the ability of sea lice to develop parasiticide resistance underpin the need to understand how these parasites respond to therapeutants used for their control (Walsh, Lyndon & Jamieson 2007). Depending on the prevailing resistance mechanism, developing biochemical assays that measure

quantitative/qualitative changes in enzymes conferring resistance, or molecular techniques (Elard, Cabaret & Humbert 1999, von Samson-Himmelstjerna & Blackhall 2005) that detect genetic changes in drug resistant parasites, is a sensible direction to follow as part of the overall parasite control strategy (Denholm et al. 2002). Genetic changes in the structure or expression of P-gp (Sangster et al. 1999) and β-tubulin (Eng et al. 2006) may provide useful markers for monitoring resistance in parasites under prolonged avermectin treatment (Eng & Prichard 2005). Selection for β-tubulin has been investigated as a marker for IVM and moxidectin resistance in nematodes (Prichard & Roulet 2007). Markers may not be directly involved in the mechanisms of resistance to be useful as long as changes in their expression are strongly associated with exposure to the specific drug. Mass use of IVM against *O. volvulus* in humans caused loss of genetic diversity of *P-gp*-like protein (Ardelli, Guerriero & Prichard 2006), and the ability to track this loss may be useful in monitoring IVM resistance (Bourguinat et al. 2008).

Investigations into differentially expressed genes in sea lice are becoming increasingly important and significant. Reverse transcription quantitative PCR (RT-qPCR) can be used to measure how a regulated gene is expressed compared with unregulated reference genes that encode proteins involved in routine cellular metabolism. However, it is important that the chosen reference gene is truly unregulated within the biological samples employed (Frost & Nilsen 2003). The study by Frost and Nilsen (2003) using RT-qPCR to identify candidate reference genes for transcription profiling throughout the life cycle of *L. salmonis* showed that the structural ribosomal protein S20 (RPS20) and the translation eukaryotic elongation factor 1α (eEF1α) show less than two fold variation in transcript levels and therefore are valid as reference genes. According to the study by Frost and Nilsen (2003), the frequently used reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could be up-regulated by up to six fold.

Although 18S RNA was highly expressed, it is not a reliable reference gene for relative quantification of most transcripts because of its relative abundance compared with RPS20, eEF1α and GAPDH, crossing the cycle threshold after 3 to 4 cycles during an RT-qPCR run (Frost & Nilsen 2003).

As the cost of developing and running molecular assays decreases and their reliability, sensitivity, and automation potential increase (Bass et al. 2004), it becomes apparent that molecular tools will become an integral and routine part of resistance detection and management in the future (Humbert et al. 2001, Sangster 2001, von Samson-Himmelstjerna 2006). This is particularly true for target-site mechanisms such as Rdl, where resistance is caused by point mutations in large and complex membrane proteins for which there are no simple biochemical assays for assessing target sensitivity (Bass et al. 2004).

Progress in developing methods and the various approaches employed in genetic studies are also very important if host-parasite interactions are to be understood. Then, new prophylactic or therapeutic strategies can be developed to arrest the propagation of sea lice.

1.6. Current investigation

1.6.1. The problem

There are reports of sea lice resistance to EMB on fish farms in the Bay of Fundy, NB (Westcott et al. 2008). Nevertheless, some sea lice populations within this region show more sensitivity to EMB compared with sea lice populations at other salmon farm locations within the Bay (Jones et al. 2012). This emphasizes the need for continual resistance monitoring so that changes in sensitivity can be detected at an early stage. Sea lice bioassays are commonly used in diagnosing clinical resistance, but cannot be routinely performed due to their lack of rapidity and simplicity. Furthermore, results of bioassays can differ widely based on time of year and site of

the parasite collection. Thus, no single bioassay technique will be suitable for all therapeutants used for sea lice control due to differences in the characteristics, stage specificity (Westcott et al. 2008, Heumann et al. 2012) and speed/duration of action of the drugs (Denholm et al. 2002). Since the bioassay must be performed shortly after detaching the parasite from the host, to avoid biased endpoints from stressed sea lice, endpoints can be unclear especially between weak and moribund parasites following exposure in this system (Denholm et al. 2002). These factors create the need for alternative methods of monitoring resistance development in the parasite, for example, using molecular tools such as RT-qPCR. Identification and monitoring expression of resistance-associated genes can assist to detect the development of resistance and to modify treatment strategies (Eng & Prichard 2005). Macrocyclic lactones can induce over-expression of P-gp in parasites (Lespine et al. 2012) and changes in the expression of this ABC transporter could be monitored as a means of detecting the development of EMB resistance in *L. salmonis* on salmon farms.

There are ongoing efforts towards impeding the rate at which EMB resistance develops in sea lice populations through the use of integrated management strategies to control the parasite in salmon farms. This includes the combined use of drugs with differing modes of action, chemicals, and non-chemical alternatives such as manipulation of host immunity using vaccines and/or immunostimulants (Jenkins et al. 1992, Raynard et al. 1994, Raynard et al. 2002). However, the effect of host immunostimulation and subsequent EMB treatment on mRNA levels of P-gp is not known. This is because inducing the innate immune response of the salmon may have unintended consequences on other treatment regimens.

The hypothesis for the present study is that P-gp is involved in resistance development to EMB in sea lice.

1.6.2. Specific objectives of the current investigation

The objectives of the present study were:

1) Determine possible links between *P-gp* and EMB by identifying whether EMB interacts with the ABC transporter;
2) Explore the use of RT-qPCR as a tool for monitoring resistance development to EMB in *L. salmonis*;
3) Identify whether P-gp mRNA expression analysis tracks resistance development in archived *L. salmonis* samples;
4) Ascertain possible enhancement of EMB efficacy through prior host immunostimulation;
5) Investigate the effects of the host innate immune response on P-gp mRNA expression in the attaching *L. salmonis* following subsequent EMB treatment;
6) Identify whether reports of EMB treatment success in Grand Manan, Bay of Fundy, NB, can be explained through EMB bioassay and P-gp mRNA expression studies;
7) Determine if other populations of sea lice, not under EMB selective pressure, display a similar or differential response to EMB bioassay assessment, such as the Grand Manan sea lice population; and
8) Investigate whether any differences identified between *L. salmonis* populations in the Bay of Fundy are heritable and result in differences in EMB sensitivity *in vivo* and *in vitro* using multi-generations and crosses of the parasite.

1.7. References

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CHAPTER 2

ROLE OF P-GLYCOPROTEIN IN EMAMECTIN BENZOATE (SLICE®) RESISTANCE IN SEA LICE, *LEPEOPHTHEIRUS SALMONIS*

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(O. Igboeli designed and carried out the experiments, analyzed the results and wrote the chapter; J. Heumann identified the *L. salmonis* P-glycoprotein mRNA sequence; J.F. Burka and M.D. Fast supervised the work and the writing)

2.1. Abstract

Emamectin benzoate (EMB; SLICE®) has been the drug of choice for the control of sea lice in salmon aquaculture within the past decade due to its ease of administration as well as efficacy on all parasitic stages of sea lice. This over-reliance has led to increased tolerance to the drug and a consequent decline in its use. ATP-binding cassette (ABC) transporters such as P-glycoprotein (*P-gp*) are known to be involved in drug resistance. The present study investigated 1) the interaction of EMB with *P-gp*, 2) the effect of increasing EMB concentrations on P-gp mRNA expression in male and female sea lice, *Lepeophtheirus salmonis,* from Atlantic salmon (*Salmo salar*) farms in the Bay of Fundy, NB, as well as 3) changes in the mRNA expression of the transporter in archived adult female *L. salmonis*. Analysis of bioassay results indicated a 4 to 26 fold higher EMB EC_{50} for samples collected in 2011 compared with a similar study carried out between 2002 and 2004 suggesting loss of EMB efficacy in the parasite. An assay for ATPase activity as well as a competitive inhibition test showed that EMB interacts with the transporter. Emamectin benzoate had a significant concentration-dependent effect on P-gp mRNA expression in the parasite. There was a temporal increase in levels of P-gp mRNA in sea lice samples collected from 2002 to 2011. Our results indicate that EMB is a substrate for *P-gp* and that the transporter could be involved in the loss of efficacy of the parasiticide in *L. salmonis*.

2.2. Introduction

Lepeophtheirus salmonis is one of the major species of sea louse that infest both wild and farmed Atlantic salmon, *Salmo salar*, (Burka, Fast & Revie 2012) and is currently the greatest challenge to profitable commercial salmon aquaculture in North America and Europe (Costello 2009). The adult parasite will normally feed on mucus, but may also ingest blood following damage of superficial epidermal capillaries exposed by extensive abrasion of the skin (Bron et al. 1993). The attachment and feeding activities of the parasite cause stress to the host (Fast, Ross & Johnson 2005) as well as osmoregulatory problems and secondary bacterial infections (Pike & Wadsworth 1999). These will lead to high treatment costs, reduced growth, reduced food conversion rate and, consequently, reduced profit margin for the salmon farmer (Costello 2009).

Drugs used for the control of sea lice are either administered orally in the feed, e.g emamectin benzoate (EMB), or topically as a bath treatment, e.g deltamethrin and azamethiphos (Burridge et al. 2010). Emamectin benzoate, a macrocyclic lactone (ML), has been one of the most effective drugs (Stone et al. 1999) for combating *L. salmonis* infection in the past decade. It is administered in salmon feed as the chemotherapeutant SLICE® at a dose of 50 μg kg^{-1} fish biomass for 7 days (Stone et al. 1999). When fed to fish, it is absorbed from the gut and distributed throughout the fish with the least and highest concentrations in the muscle and mucus, respectively (Sevatdal et al. 2005). *Lepeophtheirus salmonis* ingests EMB while feeding on the host mucus and the drug acts to block nerve transmission leading to flaccid paralysis and death of the parasite (Burka, Fast & Revie 2012). Unlike bath treatments, which are more labor intensive and can be stressful to the fish, EMB is easily administered even during unfavourable weather conditions (Stone et al. 2000). It has high efficacy against all parasitic stages of the louse, disrupting the life cycle at multiple points (Stone et al. 1999). Its effectiveness and

advantages over other sea lice parasiticides made it the preferred drug for the control of the parasite in salmon farms in the Bay of Fundy, NB (Westcott, Hammell & Burka 2004), and elsewhere, leading to concerns over resistance development (Westcott et al. 2008).

Drug resistance is an evolutionary adaptative process whereby susceptible parasites are eliminated causing resistant survivors to multiply and become the dominant population. This has been proposed for resistance development by sea lice to anti-parasitic drugs (Denholm et al. 2002). The speed at which resistance develops and its extent depend on such factors as the nature of parasite-induced damage to the host, the mechanism of resistance, frequency of drug use or selection pressure, and the parasite's biology. In a study by Bravo, Sevatdal & Horsberg (2008), over reliance on EMB was the major cause of the loss of sensitivity to another species of sea louse, *Caligus rogercresseyi,* in Chilean salmon aquaculture. Several years of use of another ML, ivermectin, prior to the introduction of EMB, also favored resistance development towards this class of drugs, suggesting lack of selectivity among the MLs.

P-glycoprotein (*P-gp*), also known as ABCB1, a member of the ATP-binding cassette (ABC) transporter protein superfamily has been linked to ivermectin resistance in nematodes (Xu et al. 1998, Eng & Prichard 2005). These transporters function by causing the efflux of chemically diverse substances from within the cell to the outside. Extrusion of lipophilic chemicals from the cell by *P-gp* is powered by ATP hydrolysis, and this can be employed to determine drugs that interact with the transporter using the ATPase activity assay (Schwab et al. 2003, Lespine et al. 2007). It is hypothesized that over-expression of P-gp, most-likely in the gut epithelium, will impede the absorption of various compounds, including EMB, ingested by the salmon louse (Tribble, Burka & Kibenge 2007). Furthermore, a novel *L. salmonis* P-gp SL-PGY1 (GenBank accession no. HQ684737) was recently cloned by Heumann et al. (2012).

There have been reports of sea lice tolerance to EMB on fish farms in New Brunswick, Canada (Westcott et al. 2010), but whether *P-gp* is involved in reduced EMB efficacy in the parasite is largely unknown. Early detection of changes in the sensitivity of sea lice towards EMB using drug resistance monitoring techniques should be a key component of a successful parasiticide resistance management plan (Westcott et al. 2008). Drug resistance monitoring should be precise, easy to perform, simple and repeatable. Sea lice bioassays are commonly used in diagnosing clinical resistance, but cannot be routinely performed due to their lack of rapidity and simplicity. Since the bioassay must be performed shortly after detaching the parasite from the host, to avoid biased endpoints from stressed sea lice, endpoints can be unclear especially between weak and moribund parasites following exposure in this system (Denholm et al. 2002, Westcott et al. 2008). Results of bioassays can differ widely based on time of year and site of the parasite collection. Also, no single bioassay will be suitable for all therapeutants used for sea lice control due to differences in the characteristics, stage specificity and speed/duration of action of the drugs (Denholm et al. 2002). These factors create the need for alternative methods of monitoring resistance development in the parasite, for example, using such molecular tools as reverse transcription quantitative PCR (RT-qPCR). Identification and monitoring expression of resistance-associated genes can assist to detect the development of resistance and to modify treatment strategies (Eng & Prichard 2005). Macrocyclic lactones can induce over-expression of P-gp in parasites (Lespine et al. 2012) and evidence for the involvement of such a gene in resistance development to a drug can be obtained by examining differences in expression of the gene between sensitive and resistant individuals (Williamson & Wolstenholme 2012), where available.

The objectives of the present study were 1) to investigate possible links between *P-gp* and EMB by identifying whether EMB interacts with the transporter using an assay for ATPase activity and through competitive inhibition of the efflux pump, 2) to explore the use of RT-qPCR as a tool for monitoring resistance development to EMB in *L. salmonis* through temporal and dose response analysis of P-gp gene expression, and 3) identify whether P-gp expression analysis tracks resistance development in archived *L. salmonis* samples.

2.3 Materials and methods

2.3.1. Materials

Emamectin benzoate (PESTANAL®) and chemicals used for this study were of analytical grade and purchased from Sigma-Aldrich, St. Louis, MO. Membrane preparation (SB-MDR1-PREDEASY™-ATPase, SOLVO Biotechnology) from *Sf*9 (*Spodoptera frugiperda*) was obtained from Xenotech, Lenexa, KS.

2.3.2. Parasite collection

Sample collections for sea lice bioassays were done in March (winter collection) and July (summer collection) 2011. These months were selected to identify seasonal influence on EMB efficacy (Lees et al. 2008, Westcott et al. 2008). Adult male and female *L. salmonis* were carefully detached from host Atlantic salmon from fish farms in the Bay of Fundy, NB, and brought back alive to the laboratory in cold seawater collected from the sampling site and maintained at 10°C overnight with aeration. Archived adult female *L. salmonis* (November 2002, February 2008, and February 2010) were collected in a similar manner, but were immediately flash-frozen on arrival at the laboratory and stored at -80°C prior to further processing. Adult male and female sea lice samples were also collected in December 2011 from

fish farms in the Bay of Fundy and F1 generation adult male sea lice grown in the laboratory (according to Westcott et al. 2008) for *P-gp* competitive inhibition tests. Similarly, the sea lice samples were maintained at 10°C overnight with aeration prior to use in the *P-gp* inhibition experiments.

2.3.3. Bioassay

Adult *L. salmonis* (240 male and 240 female) were selected within 12 h of the 10°C overnight storage and randomly distributed into Petri dishes with 10 sea lice per dish; each Petri dish contained either male or female *L. salmonis*. The sea lice were exposed to EMB at 0, 10, 100, 300, 1000 and 3000 ppb in 4 replicates per EMB concentration in a 24 h bioassay at 10°C. The EMB concentrations were coded and blinded to avoid biased analyses of the bioassay endpoint. Following the overnight incubation, the number of live, weak, moribund and dead lice per dish was determined according to Westcott et al. (2008) with slight modifications. 'Weak' refers to parasites that displayed poor and irregular swimming and were unable to attach to the Petri dish while 'moribund' refers to immotile parasites with twitching appendages. Percentage mortality was calculated following the 24 h bioassay and the current half-maximal effective concentration (EC_{50}) for EMB was derived from Trimmed Spearman-Kaber analysis (TSK) (Hamilton, Russo & Thurston 1977). Half-maximal effective concentration (EC_{50}) is the concentration of EMB that will cause 50% mortality (moribund and dead) of the parasite. To ascertain whether 24 h 10°C incubation had any effect on P-gp mRNA expression, 40 live adult female *L. salmonis* were flash-frozen shortly after collection while another 40 lice were incubated (10 lice per dish) for 24 h at 10°C without any treatment; the pre-incubated and post-incubated samples were compared for changes in P-gp mRNA expression. All the bioassay

survivors (none from the 3000 ppb exposure group) were stored in RNAlater® (Sigma-Aldrich) at 4°C for 24 h and then at -80°C prior to RNA extraction.

2.3.4. ATPase assay

Membrane preparations (SB-MDR1-*Sf*9, SOLVO Biotechnology) were incubated with EMB and ivermectin in separate assays, according to the manufacturer's protocol (Sarkadi et al. 1992). Briefly, the test compounds were dissolved in ethanol (2% maximum solvent concentration) to achieve 5 mM initial concentration of each test drug followed by a 4-fold serial dilution. The basic and verapamil-activated membrane suspensions as well as the KH_2PO_4 controls [0, 4 and 8 nmol inorganic phosphate (P_i) final assay concentration] were dispensed into a 96 well plate according to the manufacturer's setup such that each well contained 4 µg membrane (total) protein. Membrane suspensions were not added to the wells for phosphate calibration. The test drugs were added to the respective duplicate wells to achieve a concentration range of 0.01 to 100 µM final assay concentration. Prior to the initiation of ATPase reaction, the plate and MgATP solution were preincubated at 37°C for 10 min, MgATP was then added to each well with the exception of the wells for phosphate calibration and incubated at 37°C for 10 min. The ATPase reaction was terminated by addition of 100 µL of the supplied developer solution at room temperature. Two minutes later, 100 µL of the supplied blocker solution was added to the wells at room temperature. The plate was then incubated for 10 min at 37°C and the optical density read immediately at 620 nm using a BioTek® Synergy HT microplate reader (BioTek Instruments, Winooski, VT). The amount of P_i liberated in the presence and absence of 1.5 mM orthovanadate and relative to the KH_2PO_4 calibration curve were determined for both EMB and ivermectin and reported as the vanadate-sensitive ATPase activity (nmolP_i/mg protein/min). This is the activation assay and indicates whether the test

compounds will stimulate baseline vanadate sensitive ATPase activity. The inhibition study, performed concurrently with the activation assay, was used to determine if the test compounds will decrease maximum vanadate-sensitive ATPase activity following stimulation by verapamil, a strong activator of *P-gp* ATPase activity. P-glycoprotein substrates will stimulate baseline vanadate-sensitive ATPase activity while inhibitors of the efflux pump will decrease verapamil-stimulated maximum vanadate-sensitive ATPase activity.

2.3.5. P-glycoprotein inhibition test

In a preliminary study, adult male and female *L. salmonis* from host Atlantic salmon from fish farms were exposed in duplicate at 10 sea lice per Petri dish to increasing verapamil concentrations (0, 10, 30, 100, and 300 µM), and also to increasing verapamil concentrations with 100 or 300 ppb EMB concurrently in separate bioassays. This was done to verify any toxicity of the inhibitor on the parasite as well as to ascertain the concentration of EMB at which inhibition of the transporter will be evident. One hundred and 300 ppb EMB concentrations were chosen based on results of previous bioassays. In the subsequent experiment, adult male and female *L. salmonis* from the same sampling location were exposed to increasing verapamil concentrations (0, 1, 3, 10, and 30 µM), and also to the same range of increasing verapamil concentrations with 100 ppb EMB (adult female *L. salmonis*) or 300 ppb EMB (adult male *L. salmonis*) in concurrent but separate bioassays at 10 sea lice per Petri dish. The difference in EMB concentration used for the adult male and female sea lice is based on previous experiments showing that the male parasite is less sensitive to the drug compared with females. Also, first generation adult male *L. salmonis* grown in the laboratory (2 -10 per Petri dish in 5 replicates) were exposed to 300 ppb EMB with or without 10 µM verapamil; 10 µM of the *P-gp* inhibitor was chosen based on results of initial experiments (Table 2.3). The bioassays were conducted

according to previously described procedure and the criteria for determining the endpoints were the same.

2.3.6. RNA extraction

Total RNA was extracted from bioassay survivors and archived samples (stored at -80°C) using the RNeasy® Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with minor modifications. One (female) or two (male) *L. salmonis* (n = 5) were placed in a 5 mL plastic tube containing 1 mL Trizol and homogenized using a hand held electrical tissue homogenizer. The quality of all isolated RNA samples was verified with Experion™ RNA StdSens Chips (Bio-Rad Laboratories, Hercules, CA) for high quality (Fig. 2.1). The cut-off for acceptable RNA quality was 8 and above on the RNA integrity scale (1-10). The RNA concentration and the 260/280 nm ratio were confirmed using the Nanodrop 1000 Spectrophotometer (NanoDrop Products, Wilmington, DE). The samples were then stored at -80°C prior to further use.

2.3.7. Reverse transcription quantitative PCR

All the RT-qPCR steps were done according to standard procedures following the manufacturer's protocol. Briefly, 1 µg RNA of each sample was treated with DNase I (Invitrogen, Carlsbad, CA) and then reverse transcribed using SuperScript® III (Invitrogen). The PCR reactions were carried out using SYBR® GreenER™ qPCR SuperMix (Invitrogen) on a Rotor-Gene 3000 (Corbett Life Science, Concorde, NSW, AU). The cycling conditions were as follows: 50°C for 2 min (hold), 95°C for 10 min (initial denaturation), 45 cycles of 95°C for 15 sec (denaturing), 55°C for 15 sec (annealing) and 68°C for 20 sec (extension) steps, and then melt step of 72 to 95°C with 5 sec hold at each step. Primers targeting *L. salmonis* P-gp

(forward: TTCTACAGAATTGAAAGATCCGCACGAGTC; reverse: TACATAGTACCCGCATAGGCAAAGAAAGG) in the RT-qPCR were designed based on the SL-PGY1 sequence (Heumann et al. 2012) using the Primer 3 software. Expression of P-gp mRNA in the different samples were normalized to 4 reference genes (previously validated by Frost & Nilsen 2003) - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, translation eukaryotic elongation factor 1α (eEF1α) and structural ribosomal protein S20 (RPS20) using the geNorm software (Vandesompele et al. 2002). The geNorm analysis excluded 18S rRNA from computation of normalization factor utilized in the $2^{-\Delta\Delta Cq}$ analysis as the gene has relatively high abundance and instability compared with the other reference genes. Primers for the reference genes were: GAPDH (forward: TGATGGACCCTCAGCAAAGAA; reverse: CCAGTAGATGCAGGAATAATATTTTGTC), 18S rRNA (forward: GCAGCAGGCACGCAAATT; reverse: GATGAGTCCGGCTTCGTTATTTT), eEF1α (forward: TTAAGGAAAAGGTCGACAGACGTA; reverse: GCCGGCATCACCAGACTT) and RPS20 (forward: GCCGGTGTTTAACAATCATCAA; reverse: GGGCTTCGAGTCCTT GTATGC). The efficiency of the qPCR for each primer set was ≤115%. P-glycoprotein mRNA expression was derived from $2^{-\Delta\Delta Cq}$ analysis followed by comparison of each sample to the calibrator.

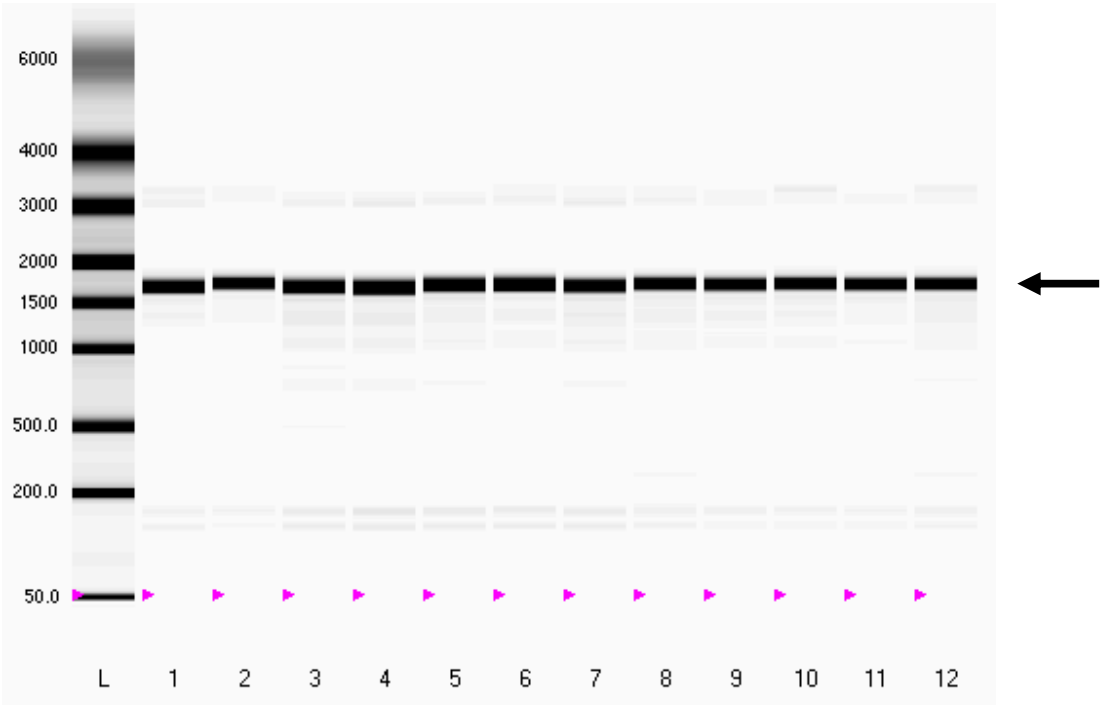

Figure 2.1. Virtual gel output for *Lepeophtheirus salmonis* RNA samples examined for integrity/degradation using Experion™ RNA StdSens Chips (Bio-Rad Laboratories, Hercules, CA). Single bands (pointed at by the black arrow) indicate high quality RNA. L = RNA ladder; numbers 1 -12 = *L. salmonis* RNA samples.

2.3.8. Statistical analysis

Relative P-gp mRNA expression was determined using one way ANOVA with the Minitab 15 statistical software (Minitab Inc., State College, PA). Statistical significance was set at $p < 0.05$. Graphical representation of the ATPase inhibition and activation assay result for EMB and ivermectin were created using the GraFit Version 7 software (Erithacus Software Ltd., Horley, UK). The remaining graphs were plotted using SigmaPlot 10.0 (Systat Software Inc., IL). The ATPase activity EC_{50} (concentration of test drug that will cause half-maximal stimulation of basal vanadate-sensitive ATPase activity) and IC_{50} (concentration of the test drug that will cause half maximal inhibition of maximum vanadate-sensitive ATPase activity) for the test compounds were determined using SigmaPlot 10.0 software.

2.4. Results

2.4.1. Bioassay

The March 2011 bioassay showed a dose-dependent effect of EMB on the survival of both the male and female *L. salmonis* (Fig. 2.2; Table 2.1). The female *L. salmonis* control group recorded a higher mean % mortality ± SEM (27.5 ± 6.3%) compared with the male control group (0%). The mean % mortality ± SEM for the male *L. salmonis* 0, 10, 100 and 300 ppb (2.5 ± 2.5, 5.0 ± 5.0 and 12.5 ± 4.8 % respectively) treatment groups were generally lower than those of the female counterparts [12.5 ± 6.3, 17.5 ± 8.5 and 45 ± 16.6% respectively (Fig. 2.2)] except for the male 1000 ppb treatment group which had a higher mean % mortality ± SEM (97.5 ± 2.5%) compared with the female counterpart (92.5 ± 4.8%).

There was also a dose-dependent EMB effect on the parasite for the July 2011 bioassay (Fig. 2.2; Table 2.2). Unlike the female control group, no mortality was recorded for the male

control group. Again, the mean % mortality ± SEM for the male *L. salmonis* treatment groups were generally lower than those of the female *L. salmonis* treatment groups with the exception of the 1000 ppb treatment groups which were 100% and 95 ± 5% for the male and female parasite respectively.

The March 2011 EMB EC_{50} values [mean ± SEM (Fig. 2.2)] derived from TSK analysis of the bioassay results were 457.20 ± 55.30 and 399.50 ± 93.70 ppb for the adult male and female *L. salmonis* respectively. These values are 1.4-1.5 fold higher than the July 2011 EC_{50} values (mean ± SEM) which were 315.30 ± 56.80 ppb for the adult male sea lice and 279.30 ± 57.20 ppb for the adult female.

2.4.2. ATPase assay

Emamectin benzoate and ivermectin stimulated baseline vanadate sensitive ATPase activity with EC_{50} of 26.35 µM (Fig. 2.3A) and 0.14 µM (Fig. 2.3B), respectively. Both compounds inhibited maximum vanadate-sensitive ATPase activity with IC_{50} of 7.82 µM for EMB (Fig. 2.3C) and 4.98 µM for ivermectin (Fig. 2.3D).

Table 2.1. Mortality analysis of adult male and female *Lepeophtheirus salmonis* following exposure to emamectin benzoate in a 24h bioassay.

Concentration of emamectin benzoate (ppb)	Mortality[@] (mean ± SEM)		% Mortality	
	Male[+]	Female[+]	Male	Female
0	0	2.75 ± 0.63	0[#]	27.5[a]
10	0.250 ± 0.250	1.25 ± 0.63	2.5[#]	12.5[a]
100	0.50 ± 0.5	1.75 ± 0.85	5[#]	17.5[a]
300	1.25 ± 0.48	4.50 ± 1.66	12.5[#]	45[a]
1000	9.75 ± 0.25	9.25 ± 0.48	97.5[*]	92.5[b]

+ Samples collected in March 2011 from Atlantic salmon farms in the Bay of Fundy, NB. Different superscript (a, b, #, *) denotes significant ($P < 0.05$) difference between two values within each gender group (male or female; n = 5). ppb = parts per billion. % = percentage. @ = moribund and dead.

Table 2.2. Mortality analysis of adult male and female *Lepeophtheirus salmonis* following exposure to emamectin benzoate in a 24h bioassay.

Concentration of emamectin benzoate (ppb)	Mortality[@] (mean ± SEM)		% Mortality	
	Male[+]	Female[+]	Male	Female
0	0	1 ± 0.41	0[#]	10[a]
10	0.25 ± 0.25	0.75 ± 0.48	2.5[#]	7.5[a]
100	0.50 ± 0.50	1.75 ± 0.479	5[#]	17.5[ab]
300	4.50 ± 1.55	5.75 ± 1.11	45[*]	57.5[bc]
1000	10	9.50 ± 0.50	100[α]	95[c]

+ Samples collected in July 2011 from Atlantic salmon farms in the Bay of Fundy, NB. Different superscript (a, b, c, #, *, α) denotes significant ($P < 0.05$) difference between two values within each gender group (male or female; n = 5). ppb = parts per billion. % = percentage. @ = moribund and dead.

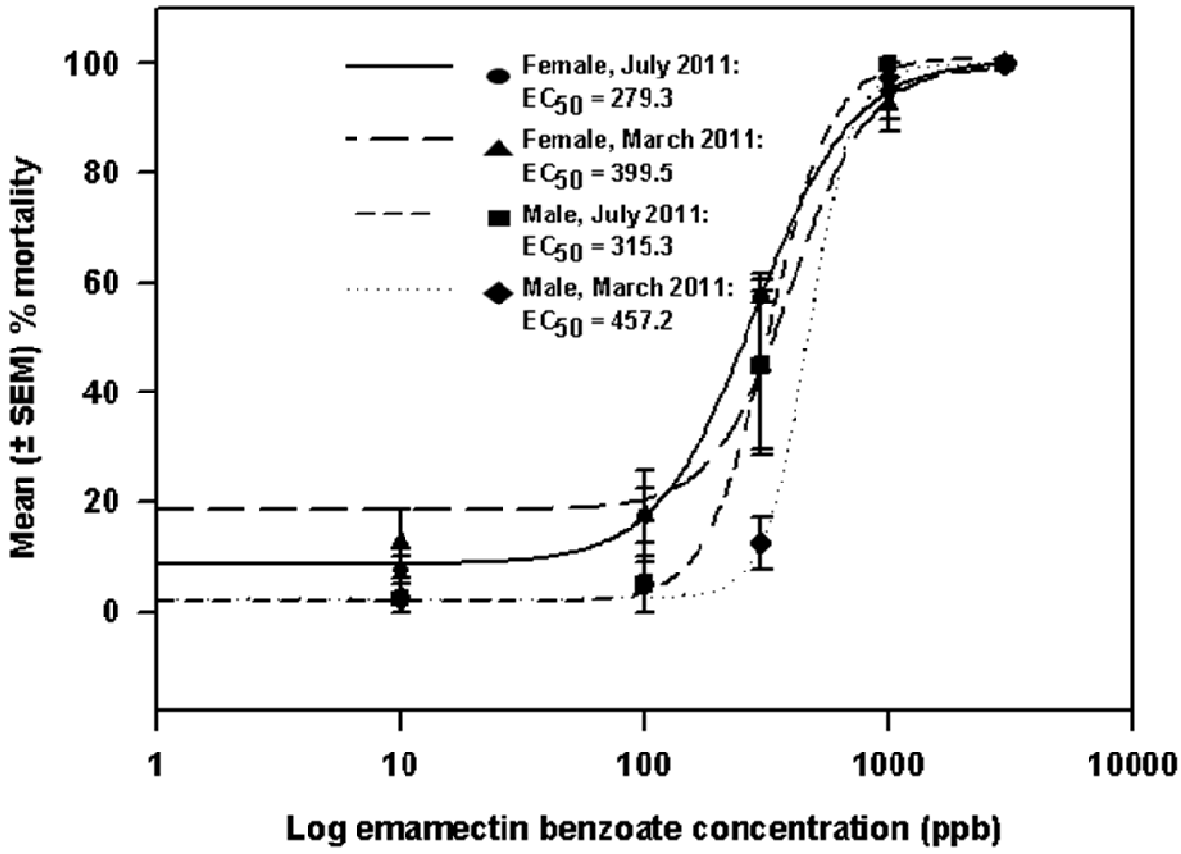

Figure 2.2. Concentration-mean (± SEM) % mortality relationship for adult male and female *Lepeophtheirus salmonis* (sea lice) exposed to emamectin benzoate in a 24 h bioassay (mean ± SEM derived from 4 replicates of 10 sea lice per EMB concentration). *Lepeophtheirus salmonis* field samples collected in March and July 2011.

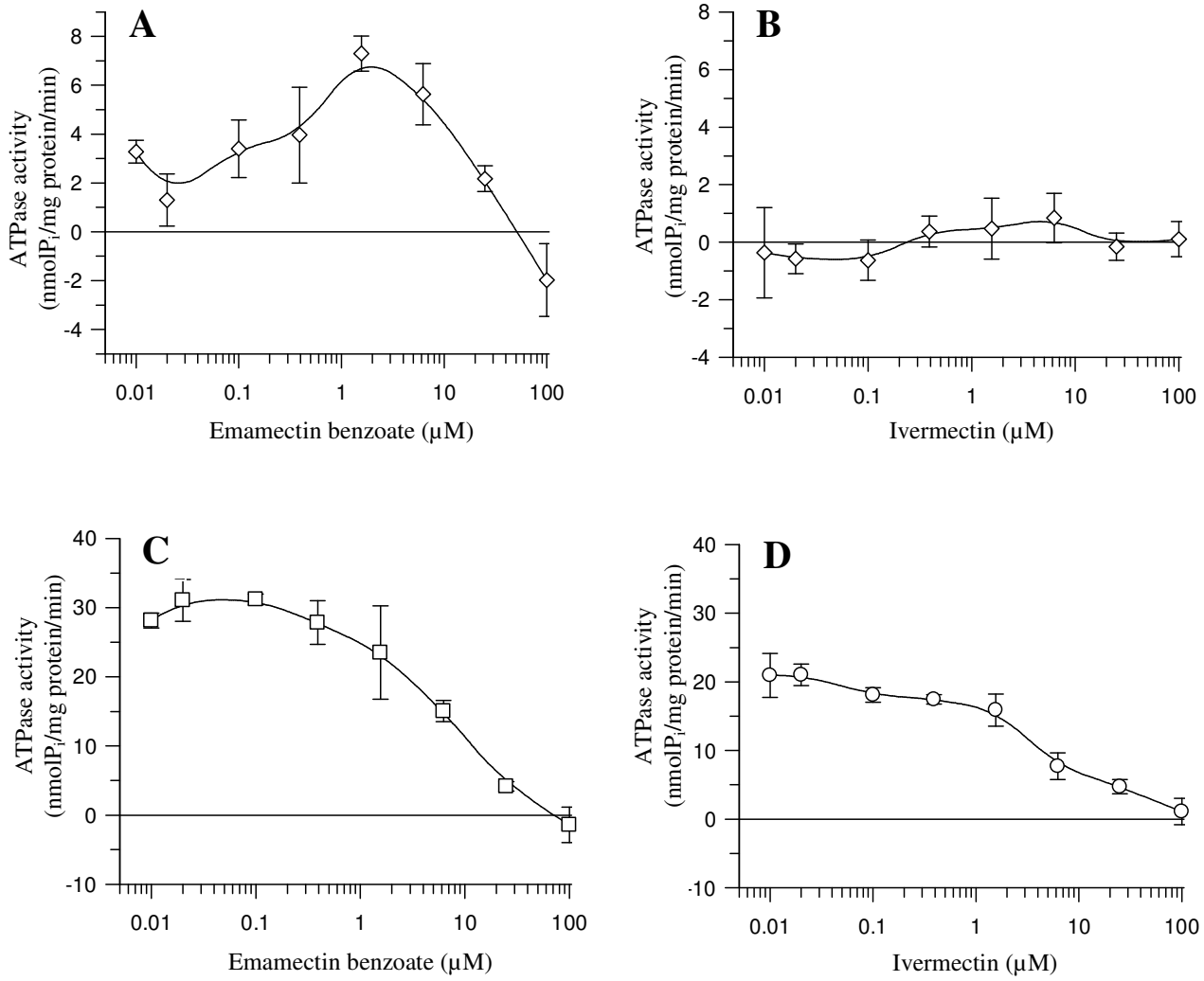

Figure 2.3. Activation (A and B) and inhibition (C and D) of ATPase activity [nmolP_i/mg protein/min (mean ± SEM); n = 4] by emamectin benzoate and ivermectin using *Sf*9 (*Spodoptera frugiperda*) membranes overexpressing P-glycoprotein (*P-gp*). Activation assay indicates whether test compounds will stimulate baseline vanadate sensitive ATPase activity; inhibition assay determines if test compounds will decrease maximum vanadate-sensitive ATPase activity following stimulation by verapamil, a strong activator of *P-gp* ATPase activity.

2.4.3. P-glycoprotein inhibition test

In the *P-gp* inhibition study, the adult male sea lice recorded less than 5% mortality for the 0, 1 and 3 µM verapamil (with or without 300 ppb EMB) treatment groups (Table 2.3). Exposure of the male parasite to 10 µM verapamil with 300 ppb EMB caused 35% mortality while exposure to 300 ppb EMB (without verapamil) resulted in 15% mortality. Also in the male sea lice, concurrent exposure to 30 µM verapamil and 300 ppb EMB caused 100% mortality while exposure to the same concentration of the parasiticide without verapamil caused 5% mortality. Percentage mortality in the female sea lice following exposure to increasing concentration of verapamil was irregular (Table 2.3). While 10 µM verapamil caused 10% mortality, simultaneous exposure to 10 µM verapamil and 100 ppb EMB caused 60% mortality for the female parasite.

For the laboratory-grown adult male sea lice, exposure to 300 ppb EMB without verapamil caused 6% mortality while exposure to the same concentration of EMB with 10 µM verapamil caused 94% mortality (Fig. 2.4).

Table 2.3. Mortality analysis of adult male and female *Lepeophtheirus salmonis* (sea lice) following exposure to emamectin benzoate (EMB) in a 24 h bioassay with or without increasing verapamil concentrations: December 2011 data (pooled sample of 20 sea lice/treatment group).

Concentration of Verapamil (µM)	% Mortality (moribund and dead)			
	Male+		Female+	
	Verapamil alone	Verapamil with 300 ppb EMB	Verapamil alone	Verapamil with 100 ppb EMB
0	0	5	20	10
1	0	0	15	5
3	0	0	30	25
10	15	35	10	60
30	55	100	65	100

+ *Lepeophtheirus salmonis* collected from Atlantic salmon farms in the Bay of Fundy, NB.

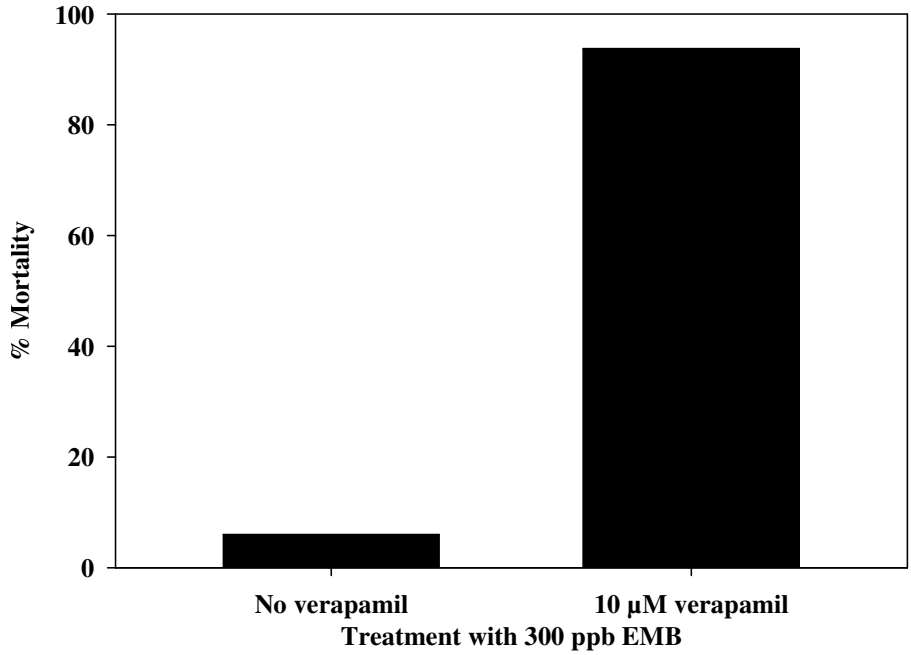

Figure 2.4. Differences in % mortality of F1 generation laboratory-grown adult male *Lepeophtheirus salmonis* (sea lice) exposed to 300 ppb emamectin benzoate (EMB) in a 24 h bioassay with and without 10 µM verapamil (pooled sample of 40 sea lice/treatment group).

2.4.4. Reverse transcription quantitative PCR

For the March 2011 bioassay survivors, EMB induced significantly higher P-gp relative mRNA expression in the male and female adult *L. salmonis* 1000 ppb treatment groups (Fig. 2.5A and 2.5B) compared with the other treatment groups within each sex category ($P < 0.05$). However, for the male *L. salmonis*, the relative P-gp mRNA expression of the 1000 ppb treatment group did not significantly differ with the 300 ppb group which also did not significantly differ from the mRNA expression levels of the transporter in the 0, 10 and 100 ppb treatment groups. For the female *L. salmonis*, the mRNA expression of the transporter in the control, 10, 100 and 300 ppb treatment groups did not significantly differ. No significant differences in P-gp relative mRNA expression were observed for the July 2011 bioassay survivors (data not shown). Overnight incubation had no effect on P-gp mRNA expression in the parasite (data not shown).

Analysis of the relative P-gp mRNA expression for the archived adult female *L. salmonis* (Fig. 2.6) revealed that the 2011 samples had a significantly higher (over 3-fold) levels compared with the samples from previous years [November 2002, February 2008 and February 2010; ($P < 0.05$)]. Although there was a general upward trend in the expression of P-gp mRNA from 2002 to 2010 (Fig. 2.6), the levels did not significantly differ.

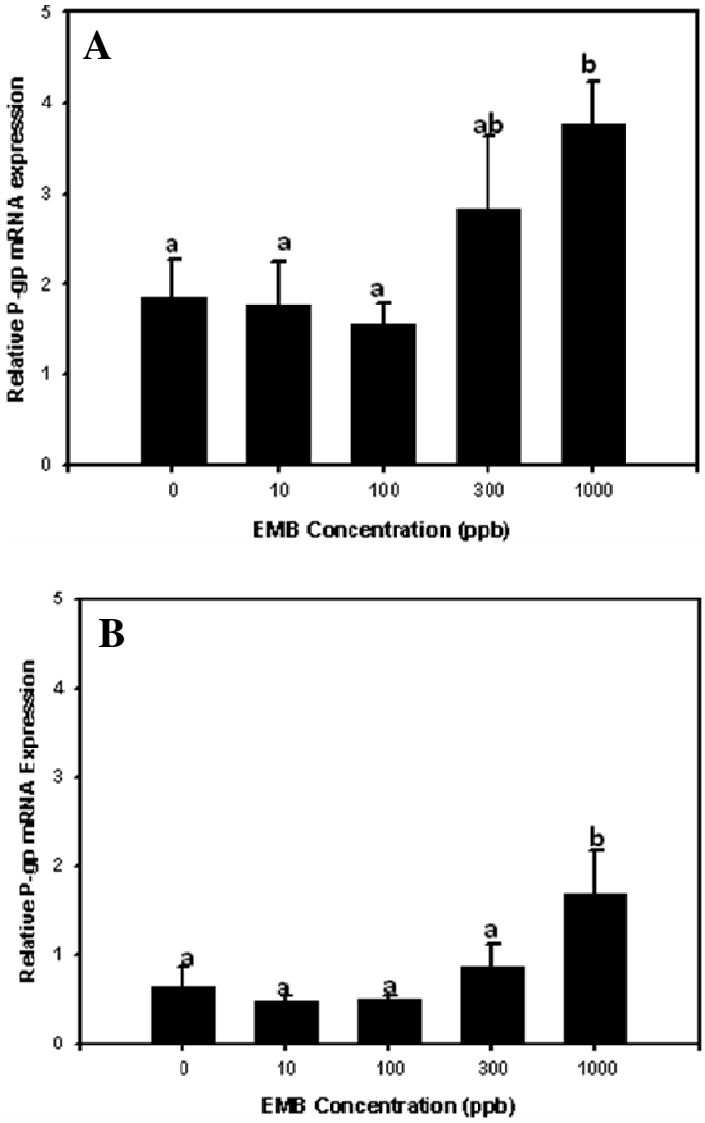

Figure 2.5. Relative P-glycoprotein mRNA expression in adult male (A) and adult female (B) *Lepeophtheirus salmonis* emamectin benzoate bioassay (EMB) survivors (mean relative expression ± SEM) normalized to 3 reference genes - (glyceraldehyde-3-phosphate dehydrogenase, translation eukaryotic elongation factor 1α, structural ribosomal protein S20) and compared with a calibrator. *Lepeophtheirus salmonis* samples collected in March 2011. A different superscript (a, b) denotes a significant ($P < 0.05$) difference between two means (n = 5).

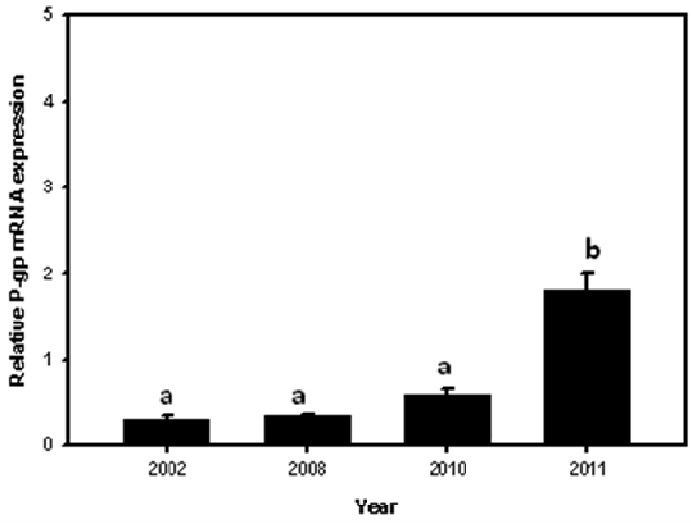

Figure 2.6. Relative P-glycoprotein mRNA expression in adult female *Lepeophtheirus salmonis* collected in November 2002, February 2008, February 2010 and March 2011 from salmon farms (mean relative expression ± SEM) normalized to 3 reference genes - (glyceraldehyde-3-phosphate dehydrogenase, translation eukaryotic elongation factor 1α, structural ribosomal protein S20) and compared with a calibrator. A different superscript (a, b) denotes a significant ($P < 0.05$) difference between two means (n = 5).

2.5. Discussion

Macrocyclic lactones have been widely used for the control of human and veterinary parasites since 1981 (reviewed by Shoop & Soll 2002), but unfortunately resistance has been developing to this family of parasiticides (reviewed by Prichard & Roulet 2007). Mechanisms of resistance include changes in the target sites or decreased concentration of the drug at the receptor due to increased metabolism of the drug or decreased uptake/increased excretion in the parasite. It is currently unknown whether EMB undergoes any significant enzymatic breakdown within the salmon louse. However, EMB undergoes only limited metabolism in the host (Kim-Kang et al. 2004) and, if this can be extrapolated to the parasite, it would suggest that changes in metabolism may not significantly contribute to resistance. Although genetic changes in the glutamate-gated chloride channel have been associated with ML resistance (Njue et al. 2004), increased expression of the ABC transporter, *P-gp*, is widely believed to be the primary mechanism responsible for loss of parasite sensitivity to MLs (reviewed by Prichard & Roulet, 2007).

Kerboeuf and Guégnard (2011) and several other studies demonstrated that nematode *P-gp* interacts with EMB as well as other MLs, hence the interest in verifying such involvement in the salmon ectoparasite, *L. salmonis*. Resistance to parasiticides have been attributed to drug selective pressure on parasite populations whereby susceptible strains are eliminated, allowing individuals that survive therapeutic concentrations of the drug to multiply and become the dominant strain, as proposed for resistance development in sea lice (Denholm et al. 2002). For an anti-infective drug to be clinically useful, resistance needs to be monitored during treatment, and concomitant use of closely related drugs should be avoided to prevent selection for drug resistance. This has been reported for *L. salmonis* in New Brunswick (Westcott et al. 2010) and

C. rogercresseyi in Chilean salmon aquaculture where several years of use of ivermectin followed by over-reliance on EMB led to the emergence of resistant populations of the parasite (Bravo, Sevatdal & Horsberg 2008). It is therefore important to identify genes and mechanisms associated with parasiticide resistance followed by monitoring the expression level of the identified genes as well as changes to the efficacy of the parasiticide. Such a combined monitoring approach will help prevent development of drug resistance in parasites such as *L. salmonis*. Studying parasites at the gene expression level also yields information such as stage and sex-related differences in drug sensitivity that could be exploited in parasite control strategies.

Bioassays can be useful in detecting clinical resistance and (Tribble, Burka & Kibenge 2007) have previously investigated potential effects of EMB on gene expression in sea lice following a 24 h bioassay. For the March 2011 (winter) bioassay in this study, the female *L. salmonis* control group recorded slightly higher % mortality than the male *L. salmonis* counterpart but this trend was less obvious for the July 2011 (summer) bioassay (Fig. 2.2). Poor fitness is generally observed in samples collected during the colder winter period compared with the warmer summer months [pers. comm. Dr. J.D. Westcott, Centre for Aquatic Health Sciences (CAHS), AVC, Charlottetown, PE], but it is not clear as to why this was more evident in the female parasite compared with the male in the present investigation. Also, it has been reported that preadult female *L. salmonis* are probably more sensitive to EMB than the preadult males (Westcott et al. 2008, Whyte et al. 2013); this was confirmed in the current study where the EC_{50} values for the female treatment groups were lower than the values for the male treatment groups. Although the reason for the sex-based discrepancy in EMB sensitivity in the parasite is yet to be elucidated, the reproductive burden imposed on the female *L. salmonis* could be a predisposing

factor. Whether the intrinsically higher expression of P-gp mRNA in the male *L. salmonis* (Fig. 2.5A) compared with the female *L. salmonis* (Fig. 2.5B) is also a contributing factor to the sex-based differences in EMB sensitivity is unknown. Bioavailability of MLs in dogs and rats has been shown to be higher in females than males and was suggested to be likely due to differences in *P-gp* and/or other MDR transporter activity or expression levels (reviewed by Lespine et al. 2009). The 1.4 to 1.5 fold higher EMB EC_{50} for the March 2011 bioassay compared with the July 2011 bioassay could be attributed to differences in water temperature in the Bay of Fundy between the two seasons; the mean water temperature in the Bay in March and July 2011 were 2.6 and 11.2 °C, respectively (pers. comm. Dr. S.K. Whyte, CAHS, AVC, Charlottetown). Variations in seasonal temperature have been associated with seasonal variation in EMB sensitivity (Westcott et al. 2008), and Lees et al. (2008) have previously shown that seasonal temperature could be a risk factor for the outcome of EMB treatment episodes for sea lice. Since the bioassays in the present study were conducted at the same temperature, the differences in EMB EC_{50} between the March and July bioassays could be traced to differing temperature preconditioning at the sampling site. Although the mechanism of such temperature effects on the parasiticide is not yet understood, knowledge of such seasonal differences in the efficacy of EMB can be useful in the timing of treatment episodes for sea lice on salmon farms. The differences in EMB EC_{50} between the two sampling periods could also be due to variations in EMB sensitivity in sea lice populations between different salmon farms in the Bay of Fundy. Sea lice samples used in the two bioassays were collected from different sites in the Bay of Fundy and Westcott et al. (2010) as well as Whyte et al. (2013) have previously shown that sea lice sensitivity to the parasiticide can vary between different salmon farms and Bay management areas within the Bay of Fundy. When compared with the EC_{50} in the present study, the EC_{50}

values derived from a similar study by Westcott et al. (2008) between 2002 and 2004 using preadult stages of the parasite were 4 to 26 fold lower and ranged from 50.00 ± 0.00 to 107.80 ± 3.80 ppb for the male parasite and 15.00 ± 14.50 to 34.50 ± 9.35 ppb for the female parasite (mean ± SEM), and is indicative of reduced EMB potency in the bioassays. The 2002-2004 bioassays were carried out using preadult stages of the parasite, whereas the present study utilized adult stages. Whether the difference in the stage of *L. salmonis* used in the bioassays accounts for the differences in EMB EC_{50} values between both studies and the magnitude of such differences are yet to be ascertained. Notwithstanding the foregoing, we suspect that a rightward shift in the EC_{50} of the parasiticide in *L. salmonis* found on Atlantic salmon in fish farms in the Bay of Fundy has occurred. This normally occurs through drug selective pressure (Lespine et al. 2012) and has been previously demonstrated for sea lice parasitosis in salmon farms elsewhere (Lees et al. 2008, Bravo, Sevatdal & Horsberg 2008).

Typical of MLs, EMB and ivermectin inhibited basal ATPase activity; Schwab et al., (2003) previously showed that ivermectin is an inhibitor of basal *P-gp* ATPase activity. In a study by Lespine et al. (2007), abamectin, eprinomectin, doramectin, ivermectin, selamectin and moxidectin, inhibited the basal *P-gp* ATPase activity with 50% inhibition at 0.2, 0.3, 0.5, 2, 3 and 10 µM, respectively. Slowly transported substrates have been shown to inhibit basal *P-gp* ATPase activity, while substrates that are more readily transported activate the ATPase activity (Lespine et al. 2007). In this study, EMB recorded an EC_{50} of 26.35 µM (Fig. 2.3A), suggesting that it is probably a more readily transported substrate than ivermectin (EC_{50}: 0.14 µM, Fig. 2.3B) as well as the previously mentioned MLs. The difference in ivermectin EC_{50} values between the study by Lespine et al. (2007) and our study may be due to the type of membrane used in the different experiments. Whereas Lespine et al. (2007) used membranes derived from

DC-3F/ADX cells from Chinese hamster lung fibroblasts overexpressing *P-gp*, the current study utilized SB-MDR1-*Sf*9 membranes overexpressing the transporter. It has been shown that variations in basal ATPase activity can exist between different membrane preparations overexpressing *P-gp* (Lespine et al. 2007). More studies to determine the rate of EMB transportation by the efflux pump using a cell-based model (Brayden & Griffin 2008) should be done to clarify our current observations. The ATPase activity inhibition study (Fig. 2.3C and 2.3D) showed that EMB, similar to ivermectin, inhibited maximal vanadate-sensitive ATPase activity with IC_{50} of 7.82 and 4.98 µM, respectively. Emamectin benzoate and ivermectin are lipophilic compounds and are slowly transported *in vivo* and such compounds have been shown to inhibit maximal vanadate-sensitive ATPase activity. Competitive inhibitors of *P-gp* could also be substrates of the transporter (Garrigos, Mir & Orlowski 1997) and further studies are necessary to confirm whether EMB, similar to ivermectin, is a substrate of the efflux pump. Ivermectin has previously been shown to be a competitive inhibitor as well as substrate of *P-gp*, hence initial attempts to use the parasiticide as a multidrug-reversing agent in drug resistant parasites (Mottier et al. 2006) and cells (Pouliot et al. 1997).

P-glycoprotein competitive inhibition test using MDR-reversing agents such as verapamil (Lespine et al. 2012) is a well established means of determining compounds that are substrates of the efflux transporter during drug development and the concept is the basis for the reversal of *P-gp*-mediated drug resistance in anthelmintic and anticancer chemotherapy. Verapamil, a well known calcium channel blocker, caused ≥ 10% mortality in *L. salmonis* at 10 and 30 µM (Table 2.3) and 100% mortality at 100 µM (data not shown), possibly due to blockade of calcium channels in the parasite. However, concurrent exposure of *L. salmonis* to EMB and verapamil caused higher % mortality compared with exposing the parasite to the parasiticide alone (Table

2.3, Fig. 2.4). This data provides further evidence that EMB interacts with *P-gp* in the parasite and also suggests that verapamil may be interacting at the same site on the efflux pump as EMB. The increased sensitivity of *L. salmonis* to EMB following concomitant verapamil exposure is most likely a consequence of competitive inhibition of *P-gp* by the MDR-reversal agent. Inhibition of the transporter limits the efflux of the parasiticide, thereby causing an increase in the concentration of EMB in the parasite. Also, the sum of mortalities due to separate exposure of the parasite to EMB and verapamil (Table 2.3) is several fold lower than the mortality recorded following combined exposure to both drugs at the 10 and 30 µM verapamil concentrations. The sex-based differences in the concentration of verapamil and EMB combined exposure that caused over 50% mortality in the parasite further reinforces the idea that the sex-based differences in EMB sensitivity observed in the parasite could be due to differences in P-gp expression between male and female *L. salmonis*. Again, the female sea lice treatment groups displayed suboptimal fitness compared with the male parasite groups, but that notwithstanding, the effect of concomitant verapamil and EMB exposure on % mortality on both parasite sexes is evident at the 10 and 30 µM verapamil exposures. This observation is consistent with previous studies on competitive inhibition of *P-gp* to ivermectin by verapamil in rat (Alvinerie et al. 1999) and *Haemonchus contortus* (Molento & Prichard 1999), and further confirms our hypothesis that *P-gp* could be involved in reduced sensitivity of *L. salmonis* to EMB. This implies that concurrent administration of MDR-reversing agents (such as verapamil) and EMB could increase the sensitivity of sea lice to the parasiticide and bring about significant reduction in EMB-resistant parasite populations to lower acceptable numbers in salmon farms where reduced EMB efficacy has been reported. More studies are necessary to confirm the possibilty of using MDR-reversing agents in the control of sea lice in Atlantic salmon farms plagued with EMB-resistant

strains of the parasite. Verapamil was used in this study as a pharmacological tool to ascertain the role of *P-gp* in resistance development *in vitro*, but would probably not be an ideal agent to use therapeutically as it is not likely to accumulate in the skin, limiting availability to the parasite. It would also have the potential of inducing cardiac toxicity to the host salmon due to Ca^{++} channel inhibition. Other ABC-transporter inhibitors with appropriate pharmacokinetics and host and human safety parameters need to be developed.

Emamectin benzoate induced overexpression of P-gp mRNA in a concentration-dependent manner for the March 2011, but not July 2011 sampling period. The approximately 2-fold P-gp mRNA expression (March 2011 sampling) in the 1000 ppb male treatment group compared with the 0, 10 and 100 ppb treatment groups (Fig. 2.5A), as well as the dose-dependent upward trend in P-gp mRNA expression (Fig. 2.5A and 2.5B) suggest that there is a positive correlation between EMB concentration and P-gp mRNA expression. Previous studies in nematodes have linked ivermectin resistance to over-expression of P-gp and the transporter has been reported to be responsible for multidrug resistance to structurally diverse drugs and chemicals used in agriculture, medicine and veterinary medicine. The upward trend in the relative P-gp mRNA expression in archived samples (Fig. 2.6) and the fact that P-gp mRNA expression for the March 2011 samples was significantly (> 2-fold) higher than the expression for 2002, 2008 and 2010 samples suggest that P-gp mRNA expression levels was increasing over the years. Although lipophilic xenobiotics in the sampling site can induce increased expression of the transporter gene in the parasite, presence of such contaminants was not confirmed and the genetic effect of continuous use of EMB on salmon farms since 2000 could be the prevailing factor (Bravo, Sevatdal & Horsberg 2008). This pattern is similar to what was observed for the March 2011 bioassay survivors (Fig. 2.5A and 2.5B) whereby high concentration of EMB (1000

ppb) induced significantly higher P-gp mRNA expression and vice versa compared with the other treatment groups and control ($P < 0.05$). Such pattern of expression was not observed for the July 2011 bioassay, suggesting possible correlation between EMB EC_{50} values and P-gp mRNA expression in *L. salmonis*. The EC_{50} value was higher in the March 2011 sampling compared with the July 2011 sampling and there were concentration-dependent differences in P-gp mRNA expression for the earlier sampling period but not for the latter. Unfortunately, only adult female parasites were archived and analysed, otherwise, it would have been interesting to investigate the changes in the expression of the transporter in the male parasite over the same period. This is because male *L.salmonis* are less sensitive to EMB and have a higher level of P-gp expression (Fig. 2.5A and 2.5B), hence will likely be a better model for tracking development of EMB resistance compared with the female parasite.

2.5.1. Conclusion

Results of the present study, especially the *P-gp* competitive inhibition test results, strongly indicate that the efflux transporter is involved in reduced sensitivity of *L. salmonis* to EMB. This observation may further be confirmed using gene knock-out strategies such as RNA interference (RNAi) technique. Changes in the expression of resistance-associated genes such as P-gp can be monitored and used in the diagnosis of early stages of resistance development to parasiticides. Although definitive diagnosis for clinical resistance can only be derived by determining changes in EC_{50}, it is important to predict when resistance is likely to occur and plan towards forestalling it. This could be achieved by monitoring markers or genes involved in reduced efficacy to the drug using such molecular tools as RT-qPCR (Williamson & Wolstenholme 2012) and our studies have demonstrated that this molecular technique can be employed in monitoring resistance development to drugs used in aquaculture. Knowledge of the

timing for resistance development will inform necessary changes to treatment options to prevent severe treatment failure. One of the greatest challenges to chemotherapy is that resistance to currently available classes of paraciticides already exists and there is an urgent need for the discovery of new classes of drugs for the control of parasites including *L. salmonis*. Although some studies have shown that MLs interact with other ABC transporters, *P-gp* is believed to be the major resistance mechanism for this family of parasiticides (Prichard & Roulet 2007, Kerboeuf & Guégnard 2011). Comparison between EMB sensitive and resistant strains of *L. salmonis* should be carried out to verify whether *P-gp* is involved in the loss of parasite sensitivity to the drug.

Using the *P-gp* competitive inhibitor, verapamil, we have demonstrated that the efflux transporter could be playing a major role in EMB resistance in *L. salmonis*. Also, results presented showed that EMB will induce overexpression of the transporter in *L. salmonis*. Further investigation is required to confirm the extent of *P-gp* involvement in reduced EMB efficacy in *L. salmonis*, and whether this can be targeted for therapy. The use of RT-qPCR as a drug resistance monitoring tool in aquaculture should also be explored further.

2.6. References

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CHAPTER 3

IMMUNOSTIMULATION OF *SALMO SALAR* L., AND ITS EFFECT ON *LEPEOPHTHEIRUS SALMONIS* (KRØYER) P-GLYCOPROTEIN mRNA EXPRESSION FOLLOWING SUBSEQUENT EMAMECTIN BENZOATE EXPOSURE

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(O. Igboeli designed and carried out the experiments, analyzed the results, and wrote the chapter; S. L. Purcell, Laboratory Manager, technical assistance; H. Wotton and J. Poley assisted with sampling and sea lice culture; J.F. Burka and M.D. Fast supervised the work and the writing)

3.1. Abstract

Control of sea lice, *Lepeophtheirus salmonis*, on farmed Atlantic salmon, *Salmo salar*, relies heavily on chemotherapeutants. However, reduced efficacy of many treatments and need for an integrated sea lice management plan require innovative strategies. Resistance to emamectin benzoate (EMB), a major sea lice parasiticide, has been linked to P-glycoprotein (*P-gp*) expression. We hypothesized that host immunostimulation would complement EMB treatment outcome. *L. salmonis* infected Atlantic salmon were fed immunostimulatory or control feeds. Sea lice were collected for 24 h EMB bioassays one and two weeks prior to commencement of EMB treatment of the fish. Two weeks after cessation of immunostimulant-treated feed, EMB was administered at 150 µg/kg fish biomass for 7 days. The bioassay revealed stage, sex and immunstimulant related differences in EMB EC_{50}. Sea lice attached to salmon with a history of immunostimulation exhibited greater survival than those on control feeds, despite similar levels of EMB in host tissues. *L. salmonis* from salmon with a history of immunostimulation also exhibited higher P-gp mRNA expression as well as greater survivability compared with controls. Administration of immunostimulants prior to EMB treatment caused increased expression of P-gp mRNA which could have consequently caused decreased efficacy of the parasiticide.

3.2. Introduction

The sea louse (*Lepeophtheirus salmonis*), a major ectoparasite of Atlantic salmon (*Salmo salar*), in the North Atlantic (Burka, Fast & Revie 2012), causes significant economic losses to commercial salmon producers (Costello 2009). Attachment and feeding activities of the parasite cause health challenges for the salmon arising from electrolyte and ion loss, stress, and opportunistic bacterial and/or viral infections (Pike & Wadsworth 1999). Strategies to control sea lice parasitosis in salmon farms include good management practices and administration of chemicals or drugs as bath or in-feed treatment, respectively. Among the several sea lice parasiticides available, emamectin benzoate (EMB), a macrocyclic lactone (ML), has been one of the most successful. Emamectin benzoate activates glutamate-gated chloride channels of the parasite where it disrupts neurotransmission causing paralysis and, consequently, killing the sea louse (Burka, Fast & Revie 2012). The drug is easily administered and is effective against all parasitic stages of sea lice (Stone et al. 1999). Over-reliance on EMB by salmon farmers (Westcott, Hammell & Burka 2004) consequently led to resistance development to the drug by *L. salmonis* in Atlantic Canada and Europe (Hjelmervik et al. 2010, Westcott et al. 2010) and *Caligus rogercresseyi* (Bravo, Sevatdal & Horsberg 2008) in Chile.

Widespread development of drug resistance in *L. salmonis* underscores the need for an integrated sea lice management approach to the control of sea lice in salmon farms. This can be achieved through the combined use of drugs, chemicals, and non-chemical alternatives including manipulation of host immunity using vaccines and/or immunostimulants (Jenkins et al. 1992, Raynard et al. 2002). Stimulating the innate immunity of fish hosts against pathogen and parasite invasion (Bricknell & Dalmo 2005) is increasingly adopted as part of disease management in aquaculture including sea cage salmon farming (Tacchi et al. 2011).

Commonly used immunostimulants in aquaculture, β-glucan/yeast extracts (Bridle et al. 2005, Dalmo & Bøgwald 2008, Guselle et al. 2010) and a known innate stimulating compound, cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN) (Jørgensen et al. 2003, Carrington & Secombes 2006), were investigated in-feed for their ability to enhance EMB efficacy in *L. salmonis*. These immunostimulants are highly conserved pathogen-associated molecular patterns (PAMPs) found in lower organisms, but absent in Metazoa (Tsoni & Brown 2008). Whereas β-glucans occur as polysaccharides in fungi, plants and bacteria (Tsoni & Brown 2008), CpG ODNs are unmethylated motifs found within bacterial and viral DNA (Carrington & Secombes 2006). Presence of PAMPs within a host are sensed by pattern recognition receptors (PRRs) such as Toll-like (TLR) (Bricknell & Dalmo 2005, Cuesta, Esteban & Meseguer 2008, Palti et al. 2010) and C-lectin (CLR) receptors (Tsoni & Brown 2008). On recognition of PAMPs, PRRs activate signalling pathways which lead to transcription of genes involved in the immune response, including inflammation, antiviral responses and dendritic cell maturation (Whyte 2007). During sea lice parasitosis, a strong inflammatory response by salmon is necessary for early and effective rejection of the parasite (Johnson & Albright 1992, Fast et al. 2003). Hence administration of immunostimulants to Atlantic salmon to boost the innate response to sea lice infection may enable these fish to eliminate the parasite more effectively.

The basis of this study is that prior host immunostimulation followed by drug treatment will lead to greater louse mortality compared with individual use of immunostimulants or drugs. It was hypothesized that the innate immune response would likely weaken the parasite, making it more sensitive to the action of a parasiticide such as EMB. The effect of host immunostimulation and subsequent EMB treatment on mRNA expression of P-gp, a major EMB resistance mechanism in *L. salmonis* (Chapter 2), is not known. Some studies have shown that

acute and chronic inflammatory responses can increase or decrease P-gp expression, respectively (Dumoulin et al. 1997, Ho & Piquette-Miller 2006). The aims of this study were to a) ascertain possible enhancement of EMB efficacy through prior host immunostimulation and b) investigate the effects of host innate immune response on P-gp mRNA expression in the attaching *L. salmonis* following subsequent EMB treatment.

3.3. Materials and methods

3.3.1. Experimental design

Two recirculation units each consisting of 8 (330 L) tanks with parallel biofilter systems were set up and maintained at 11 (± 1) °C water temperature. Atlantic salmon smolts with a were procured from Buckman's Creek Hatchery Ltd., Pennfield, NB, and transported to the Atlantic Veterinary College (AVC) Aquatic Facility. The fish had a mean weight of 155.1 ± 82.2 g (mean ± SD) at the beginning of the study and were randomly allocated to tanks at 30 fish per tank and allowed to acclimatize to the system (freshwater) for 3 weeks. The system was then changed to recirculation and the salinity gradually raised to ~33 gL^{-1} with Instant Ocean® (Aquarium Systems, Cincinnati, OH) over 7 days. The fish were allowed to acclimatize again for 3 weeks prior to commencement of immunostimulant feed administration (Table 3.1). The fish were fed 1% body weight control diet and maintained on a 14 h light: 10 h dark cycle. Concentration of ammonia, nitrite, oxygen and nitrate in the water were closely monitored and maintained at optimal levels throughout.

3.3.2. Immunostimulant feeds

Feeds used in this study were produced by Northeast Nutrition Ltd., Truro, NS, as 2.5 mm pellets. The tanks containing the salmon were randomly assigned to the three

immunostimulants SLX, CpG and Aquate®, in duplicates; control feeds were allocated to 4 tanks, 2 tanks each for EMB treated and untreated fish. SLX is a non-commercial proprietary immunostimulant feed containing an undisclosed immunostimulant milled directly into the feed at 0.6 g/kg of feed, concentration known to induce host inflammatory processes. CpG immunostimulant feed was produced by incorporating unmethylated CpG ODN (Sigma, St. Louis, MO) into the base feed at 2 g/kg feed. The third immunostimulant feed, 0.2 % Aquate® (Stirling Products, Charlottetown, PE), contained crude yeast extracts.

3.3.3. Infection of *S. salar* with *L. salmonis* copepodids

Copepodids hatched from egg strings of EMB resistant (based on previous EMB bioassay results) adult female *L. salmonis* collected from salmon farms in the Bay of Fundy, NB, were used to infect the salmon according to the method of Covello et al. (2011). Approximately 2100 copepodids were introduced into each tank with increased aeration for about 6 h (Table 3.1). Two days later, an additional 1450 copepodids were added to each tank using the same procedure. Prior to sea lice infection, the recirculation system was turned off and a 100 µm net installed across each inflow valve of the different tanks to prevent cross-infection between tanks. Water temperature (11 ± 1°C) and O_2 level (7-9 mg L^{-1}) were monitored and maintained in each tank throughout *L. salmonis* infection period.

3.3.4. Bioassay

Two and one week(s) prior to commencement of EMB treatment (Table 3.1), fish samples from all the control and treatment groups were euthanized with a lethal dose of MS-222 (250 mg L^{-1}; Syndel Laboratories Ltd., Qualicum Beach, BC) and sea lice samples were collected. The sea lice were at preadult and adult stages at the first and second sampling, respectively. Adult sea

lice from the second sampling served as EMB untreated control for immunostimulant/EMB treament component of this study. The samples were stored overnight at 10°C in salt water (33-36 g L^{-1} salinity) and then used for EMB bioassay within 12 h of collection for both the first. The bioassay was carried out according to the described protocol in Chapter 2.

The EMB concentrations used for the first sampling (preadult stage) were 0, 10, 100, 300 and 1000 ppb while 0, 100, 200, 400 and 1000 ppb EMB were used for the second sampling (adult satge). The different range of EMB concentrations used was based on the different parasite stage used. At the end of the 24 h EMB bioassay, half-maximal effective concentration (EC_{50}) for the parasiticide was derived using US Environmental Protection Agency (USEPA) Trimmed Spearman-Kaber (TSK) software version 1.5 (Hamilton, Russo & Thurston 1977) for each treatment group. Survivors of the bioassay were flash-frozen in liquid nitrogen and immediately stored at -80°C. The EMB untreated control sea lice were analysed to verify stage-, sex-, and immunostimulant-related differences in relative P-gp mRNA expression.

3.3.5. Treatment with immunostimulants and EMB

Atlantic salmon were placed on the immunostimulant diets for 7 weeks (Table 3.1). Two weeks following cessation of immunostimulant diets, fish were placed on EMB at 150 µg/kg fish biomass for 7 days consistent with the current dose used in salmon farms in Atlantic Canada (M. Beattie, Department of Agriculture, Aquaculture and Fisheries of New Brunswick, **http://www.dfo-mpo.gc.ca/science/enviro/aquaculture/rd2011/rdsealice-pou-eng.html**) as the manufacturer's recommended 50 µg/kg fish biomass for 7 days is no longer clinically effective. Two groups of fish with no immunostimulant background served as EMB-treated and -untreated controls.

Table 3.1. Timeline in days for infection of Atlantic salmon, *Salmo salar*, with *Lepeophtheirus salmonis* copepodids, administration of different immunostimulant feeds and emamectin benzoate (EMB), and sampling of *L. salmonis*.

Days	0	19	21	49	62	63	67	70	74	84	98
Activity	Immunostimula-nt feed commenced	First infection of fish with copepodids	Second infection of fish with copepodids	Immunostim-ulant feed stopped, Fish placed on regular feed	Sea lice samples collected prior to EMB treatment	Fish placed on EMB at 150 µg/kg fish biomass	Sea lice samples collected (4 dpS)	EMB treatment ended	Sea lice samples collected (5 dpSC)	Sea lice samples collected (15 dpSC)	Final sea lice sampling (29 dpSC)

dpS = days post-commencement of EMB treatment; dpSC = days after cessation of EMB treatment.

3.3.6. Determination of EMB concentration and *L. salmonis* infection levels

Four days post-commencement (dpS) of EMB treatment (Table 3.1), 5 fish/tank (randomly selected) were euthanized with a lethal dose of MS-222 (250 mg L-1; Syndel Laboratories Ltd) to determine the level of sea lice infection, collect sea lice samples for mRNA expression analysis, as well as collect salmon muscle and skin samples to measure EMB concentration. The sampling was repeated 5, 15, and 29 days post-cessation (dpSC) of EMB treatment (Table 3.1). Sea lice samples, and salmon muscle and skin were flash-frozen (on dry ice) on collection and stored at -80°C for further studies. Determination of EMB concentration in the salmon muscle and skin was conducted according to the method of (van de Riet, J. M. et al. 2001) by the Toxicology and Analytical Services Laboratory at the AVC. Emamectin benzoate concentrations in the skin were measured in pooled, rather than individual, samples.

3.3.7. RNA extraction

Total RNA extraction was conducted following standard procedures. One (female) or two (male) *L. salmonis* per group (4 replicates) were placed in a 5 mL plastic tube containing 0.5 mL trizol reagent. The sample was then homogenized using an electric tissue homogenizer (VWR, Mississauga, ON). Following routine standard washes and precipitation, the resultant RNA pellet was resuspended in molecular grade water. Randomly selected subsets of the RNA samples were verified for quality using the Experion™ RNA StdSens Chips (Bio-Rad Laboratories, Hercules, CA). Cutoff for good quality RNA was ≥ 8 on the RNA integrity number rating. The RNA concentration and 260/280 nm ratio were determined using a Nanodrop Spectrophotometer 2000 (Thermo Scientific, Wilmington, DE); the samples were then stored at -80°C for further use.

3.3.8. Reverse transcription quantitative PCR (RT-qPCR)

The RT-qPCR steps were conducted according to standard procedures and have been described elsewhere (Chapter 2) with slight modifications. Briefly, 1 µg RNA from each sample was treated for DNA contamination using TURBO DNase-*free*™ kit (Ambion, Carlsbad, CA) and then reverse transcribed using Reverse Transcription System (Promega, Madison, WI), according to the manufacturer's instructions. DNA elimination was confirmed in qPCR using reverse transcriptase-omitted controls. The qPCR reactions were perfomed using GoTaq® qPCR Master mix (Promega) and were run on the Realplex thermocycler (Eppendorf, Mississauga, ON). Expression data normalization was conducted using 4 reference genes employed in the preceeding study (Chapter 2) - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, translation eukaryotic elongation factor 1α (eEF1α), and structural ribosomal protein S20 (RPS20) - using geNorm software (Vandesompele et al. 2002). The primers for the different genes were the same as reported in Chapter 2; the reaction efficiency (%) for each primer set were ≤106 (Table 3.2). The cycling conditions for P-gp were 95°C for 10 min (initial denaturation), 40 cycles of 95°C for 15 sec (denaturing), 55°C for 15 sec (annealing) and 68°C for 20 sec (extension) steps, and melt step of 60°C for 15 sec to 95°C for 15 sec to confirm amplification single PCR product. The cycling conditions for the the 4 reference genes were same as P-gp but for the cycling step which was 40 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 15 sec. Relative P-gp mRNA expression was determined using the $2^{-\Delta\Delta Cq}$ method normalized to the calibrator. 18S rRNA was excluded from geNorm computation of normalization factor utilized in the $2^{-\Delta\Delta Cq}$ analysis due to its relatively high abundance and level of instability compared with the other reference genes.

Table 3.2. Primer sets used in qPCR experiments to amplify reference (GAPDH, 18S rRNA, eEF1α and RPS20) and target (P-gp) genes in *Lepeophtheirus salmonis*.

Gene	Primer sequence 5'→3'	Reference	Reaction efficiency (%)
SL-PGY1 (P-gp)	Forward: TTCTACAGAATTGAAAGATCCGCACGAGTC Reverse: GTACATAGTACCCGCATAGGCAAAGAAAGG	Present study	101
GAPDH	Forward: TGATGGACCCTCAGCAAAGAA Reverse: CCAGTAGATGCAGGAATAATATTTTGTC	(Frost & Nilsen 2003)	100
18S rRNA	Forward: GCAGCAGGCACGCAAATT Reverse: GATGAGTCCGGCTTCGTTATTTT	(Frost & Nilsen 2003)	99
eEF1α	Forward: TTAAGGAAAAGGTCGACAGACGTA Reverse: GCCGGCATCACCAGACTT	(Frost & Nilsen 2003)	100
RPS20	Forward: GCCGGTGTTTAACAATCATCAA Reverse: GGGCTTCGAGTCCTT GTATGC	(Frost & Nilsen 2003)	106

P-gp = P-glycoprotein; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; eEF1α = translation eukaryotic elongation factor 1α; RPS20 = structural ribosomal protein S20.

3.3.9. Statistical analysis

Relative expression of P-gp mRNA was determined by performing multivariate analysis of variance (MANOVA) using the STATISTICA statistical software (StatSoft Inc., Tulsa, OK). A Tukey honestly significant difference post-hoc analysis was also conducted using STATISTICA to determine significant differences in P-gp mRNA expression among the samples. Statistical significance was set at $p<0.05$ and ≥ 1.5 fold differences. Graphical representations of relative P-gp mRNA expression and salmon muscle concentration of EMB were plotted using SigmaPlot 10.0 (Systat Software Inc., Chicago, IL).

3.4. Results

3.4.1. Bioassay

Stage, sex, and immunostimulant-related differences in EMB EC_{50} were revealed following TSK analysis of bioassay results (Table 3.3). While EMB EC_{50} values for male sea lice increased ~2-3 fold from pre-adult to adult stage, female EC_{50} values were within the same range (171to 342ppb) for these life stages. We were unable to derive confidence intervals (CI) for immunostimulant-untreated, SLX and CpG groups. Also, EMB EC_{50} values for the male Aquate® group was incaculable because only 40% mortality occurred for this group at the highest EMB concentration used (1000 ppb) (Table 3.3). There was ≤ 20 % mortality for both sexes in the preadult stages and in the female adult sea lice EMB-untreated controls, but no mortality in the adult male EMB untreated controls (data not shown).

3.4.2. Emamectin benzoate concentration in *S. salar* muscle and skin

The concentration of EMB in the salmon muscle significantly differed among the different immunostimulant treatment groups on 4 dpS and 5 dpSC ($P < 0.05$), but not on 15

dpSC (Fig. 3.1). At 4 dpS, muscle EMB concentrations for the control group receiving no immunostimulant and the group fed CpG diets, 144.0 ± 21.2 and 142.1 ± 12.7 ppb (mean ± SEM), respectively, were significantly lower than the concentration of the parasiticide in the SLX- and Aquate®-treated fish, which were 195.6 ± 19.2 and 234.1 ± 39.2 ppb, respectively ($P < 0.05$). Emamectin benzoate was detected in fish from one of the two replicate untreated control tanks, just above the detection limit at 4 dpS and 5 dpSC; no sample was assessed for this group at15 dpSC (Fig. 3.1). The highest EMB concentrations were recorded at 4 dpS and 5 dpSC, 234.1 ± 39.2 and 231.2 ± 21.5 ppb, respectively, for the Aquate® treatment group.

The EMB concentration in the skin of EMB-treated fish ranged from 225.8 (Aquate®) to 270.8 (SLX) ppb at 4 dpS with the EMB control and CpG fish having intermediate values of 250.0 and 231.7 ppb, respectively (data not shown). Again, the parasiticide was detected in the EMB untreated control at 5 and 15 dpSC, 4.8 and 16.5 ppb EMB, respectively; samples were not assessed for the control group at 4 dpS and 29 dpSC. Peak EMB concentrations for the different treatment groups were recorded at 5 dpSC. The highest EMB concentration was recorded for Aquate® (539.2 ppb) followed by EMB treated control, CpG and SLX with EMB concentrations of 453.3, 383.0 and 318.9 ppb, respectively. By 29 dpSC, EMB concentration in the skin ranged from 90.8 (SLX) to 102.8 (EMB treated control) ppb for the different treatment groups.

3.4.3. *Lepeophtheirus salmonis* infection levels

The % survival of adult male sea lice was higher than that of females for the different control and treatment groups (Table 3.4). Percentage survival refers to the percentage of time 0 sea lice numbers that survived to 29 dpSC. All groups exhibited <60% survival across both sexes by the end of the study, with immunostimulant fed groups showing highest % survival (Table 3.4).

3.4.4. *L. salmonis* P-glycoprotein mRNA expression

There were stage, sex and immunostimulant background related differences in relative P-gp mRNA expression (Fig. 3.2 A and B) of the EMB untreated control bioassay survivors collected prior to commencement of triple dose EMB treatment. Relative P-gp mRNA expression in male sea lice did not significantly differ among all the treatment groups with the exception of the adults exposed to salmon fed Aquate® diet (Fig. 3.2A) which was significantly higher than SLX (preadult and adult) and CpG (preadult) groups ($P < 0.05$). P-glycoprotein mRNA expression was significantly higher in pre-adult females compared with adults with the exception of adult sea lice with SLX, CpG and Aquate® immunostimulant (Fig. 3.2B, $P < 0.05$). Sea lice from the three latter groups recorded significantly lower P-gp mRNA expression compared with pre-adult sea lice associated with the Aquate® feed group ($P < 0.05$).

Male *L. salmonis* showed an increase in the expression of P-gp mRNA at 4dpS and 5dpSC relative to pre-EMB treatment levels (day 0) for the different treatment groups and controls (Fig. 3.3A). P-gp mRNA expression was low prior to EMB treatment and at 15 and 29 dpSC compared with 4 dpS and 5 dpSC.

Again there was an increase in the female P-gp mRNA expression for sea lice from all the groups and controls from a low expression prior to EMB treatment to high expression at 15 dpSC and then down to an intermediate level by 29 dpSC. Sea lice from the Aquate® diet group recorded a significantly higher P-gp mRNA expression ($P < 0.05$) at 15 dpSC compared with the other sampling periods and to the other treatment groups at all the sampling time points with the exception of SLX at 15 dpSC (Fig. 3.3B). While the peak adult male sea louse P-gp mRNA expression was recorded for SLX at 5 dpSC (Fig. 3.3A), the highest expression of the transporter for females was recorded for Aquate® treatment group at 15 dpSC (Fig. 3.3B).

Table 3.3. Half-maximal effective concentration (EC_{50}) (95% confindence interval) for preadult and adult *Lepeophtheirus salmonis* exposed to increasing concentrations of emamectin benzoate (EMB).

Stage	Sex	Treatment	% mortality at 1000 ppb EMB	EMB EC_{50} (ppb) (95% CI)
Pre-adult[a]	Male	No Tx Ctrl	100	308 (214, 443)
		SLX	100	373 (264, 525)
		CpG	100	288 (201, 414)
		Aquate®	100	291 (180, 471)
	Female	No Tx Ctrl	100	250 (178, 352)
		SLX	100	319 (211, 484)
		CpG	100	281 (180, 471)
		Aquate®	100	207 (112, 381)
Adult[b]	Male	No Tx Ctrl	50	1000
		SLX	50	1000
		CpG	100	632
		Aquate®	40	-
	Female	No Tx Ctrl	100	171 (118, 248)
		SLX	100	219 (177, 270)
		CpG	100	342 (259, 451)
		Aquate®	100	292 (214, 397)

Sea lice samples were first generation laboratory reared *L. salmonis* associated with different immunostimulant (SLX, CpG or Aquate®) diets. No Tx Ctrl = no immunostimulant treatment. Half-maximal effective concentration (EC_{50}) is the concentration of EMB that will cause 50% mortality (moribund and dead) of sea lice.
[a]Sea lice exposed to 0, 10, 100, 300 and 1000 ppb EMB.
[b]Sea lice exposed to 0, 100, 200, 400 and 1000 ppb EMB.
- = not determined (40% mortality at maximum EMB concentration).

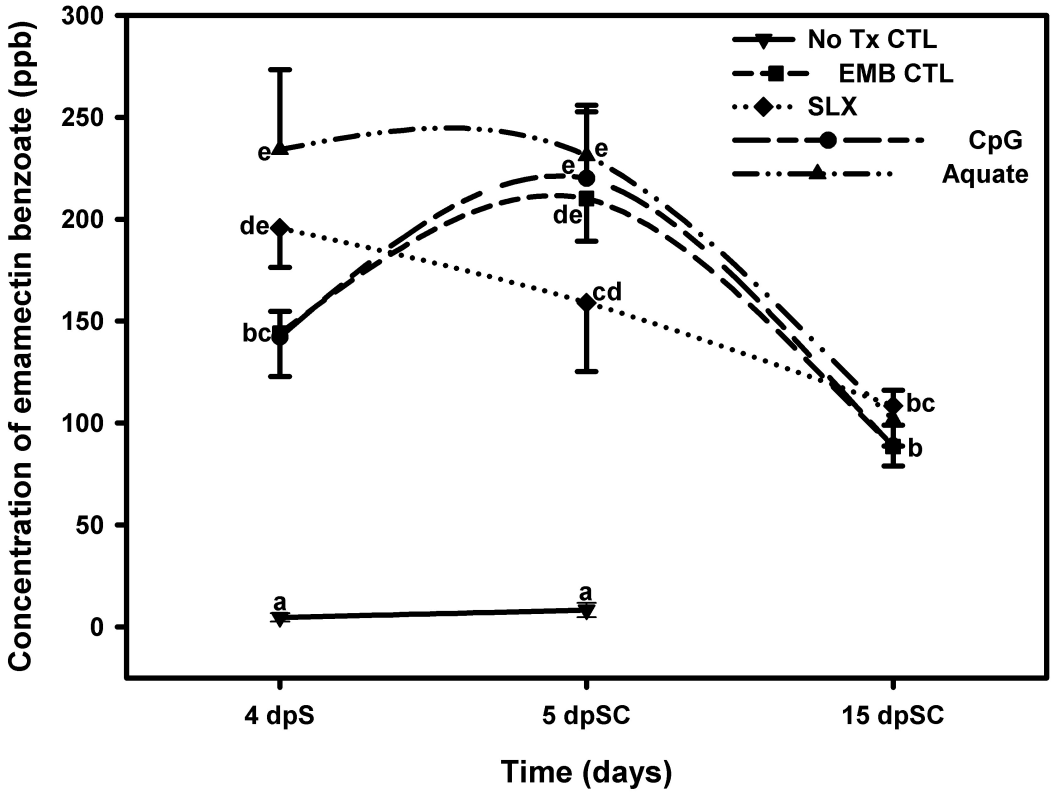

Figure 3.1. Concentration of emamectin benzoate (EMB) in muscle of Atlantic salmon following administration of different immunostimulant (SLX, CpG or Aquate®) diets fed for 7 weeks. No Tx CTL = no immunostimulant treatment; EMB CTL = EMB treatment only; dpS = days post-commencement of EMB treatment; dpSC = days after cessation of EMB treatment. Different superscripts (a, b, c, d, e) denote significant ($P < 0.05$) differences between two means (n=5).

Table 3.4. Infection levels of adult *Lepeophtheirus salmonis* on Atlantic salmon before, during, and following emamectin benzoate (EMB) treatment.

Treatment group	Time, sex and ± SEM sea lice numbers per fish (n = 5)										% survival at 29 dpSC	
	0		4 dpS		5 dpSC		15 dpSC		29 dpSC			
	M	F	M	F	M	F	M	F	M	F	M	F
Control	13.3 ± 1.4	14.1 ± 1.5	5.0 ± 0.7	8.4 ± 1.2	2.6 ± 0.7	6.1 ± 1.2	4.1 ± 0.4	1.6 ± 0.6	2.1 ± 0.5	0.8 ± 0.5	15.8	5.7
EMB Ctl	10.6 ± 1.9	8.8 ± 1.8	4.2 ± 0.7	5.5 ± 1.1	1.8 ± 0.4	3.8 ± 0.9	4.8 ± 0.7	5.0 ± 1.3	2.8 ± 0.6	2.0 ± 0.5	26.4	22.8
SLX	11.8 ± 2.3	15.6 ± 3.0	7.6 ± 1.3	9.1± 1.6	3.1 ± 0.8	8.4 ± 1.0	6.8 ± 0.8	6.2 ± 0.8	6.8 ± 1.1	4.6 ± 1.1	57.7	29.6
CpG	8.4 ± 1.4	11.7 ± 1.9	5.5 ± 0.9	7.5 ± 1.7	2.2± 0.7	5.7 ± 1.6	5.3 ± 1.1	5.5± 1.8	4.9 ± 1.2	4.4 ± 1.2	58.1	37.7
Aquate®	7.6 ± 0.9	6.9 ± 0.9	4.6 ± 0.9	4.3± 1.3	2.1 ± 0.5	4.0 ± 1.0	3.3 ± 0.6	1.7 ± 0.5	4.4 ± 1.2	1.8 ± 0.8	58.7	25.8

Sea lice samples are first generation laboratory reared *L. salmonis* associated with different immunostimulant (SLX, CpG or Aquate®) diets. EMB Ctl = EMB treatment only; dpS = days post-commencement of EMB treatment; dpSC = days after cessation of EMB treatment; % survival = percentage of 0 time *L. salmonis* numbers compared with 29 dpSC sea lice numbers.

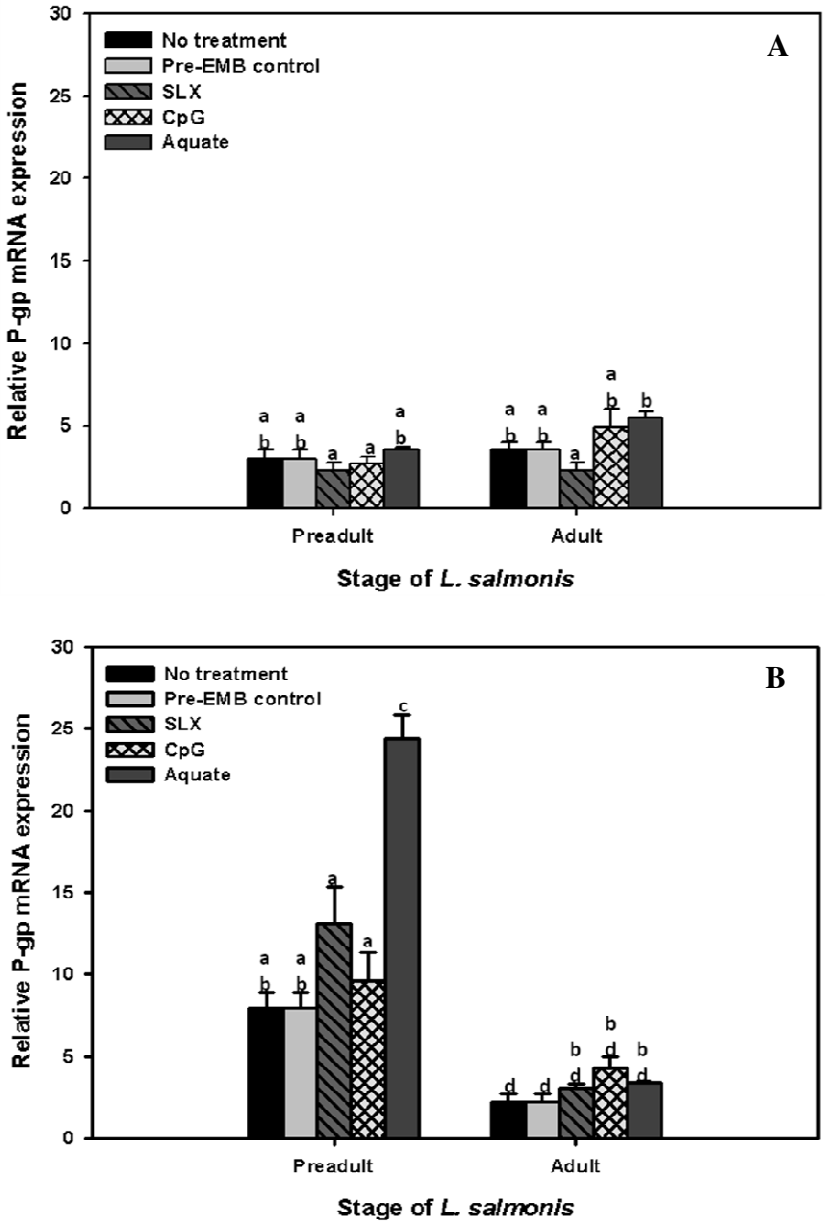

Figure 3.2. Relative P-glycoprotein mRNA expression in pre-adult and adult male (A) and female (B) *Lepeophtheirus salmonis* emamectin benzoate bioassay (EMB) untreated survivors (mean relative expression ± SEM) normalized to 3 reference genes - (glyceraldehyde-3-phosphate dehydrogenase, translation eukaryotic elongation factor 1α, structural ribosomal protein S20) and compared with a calibrator. *Lepeophtheirus salmonis* samples are first generation laboratory reared sea lice associated with different immunostimulant (SLX, CpG or Aquate®) diets. No Tx = no immunostimulant treatment; Pre-EMB CTL = prior to EMB treatment/also not treated with immunostimulants. Different superscripts (a, b, c, d) denote significant ($P < 0.05$) differences between two means (n = 6).

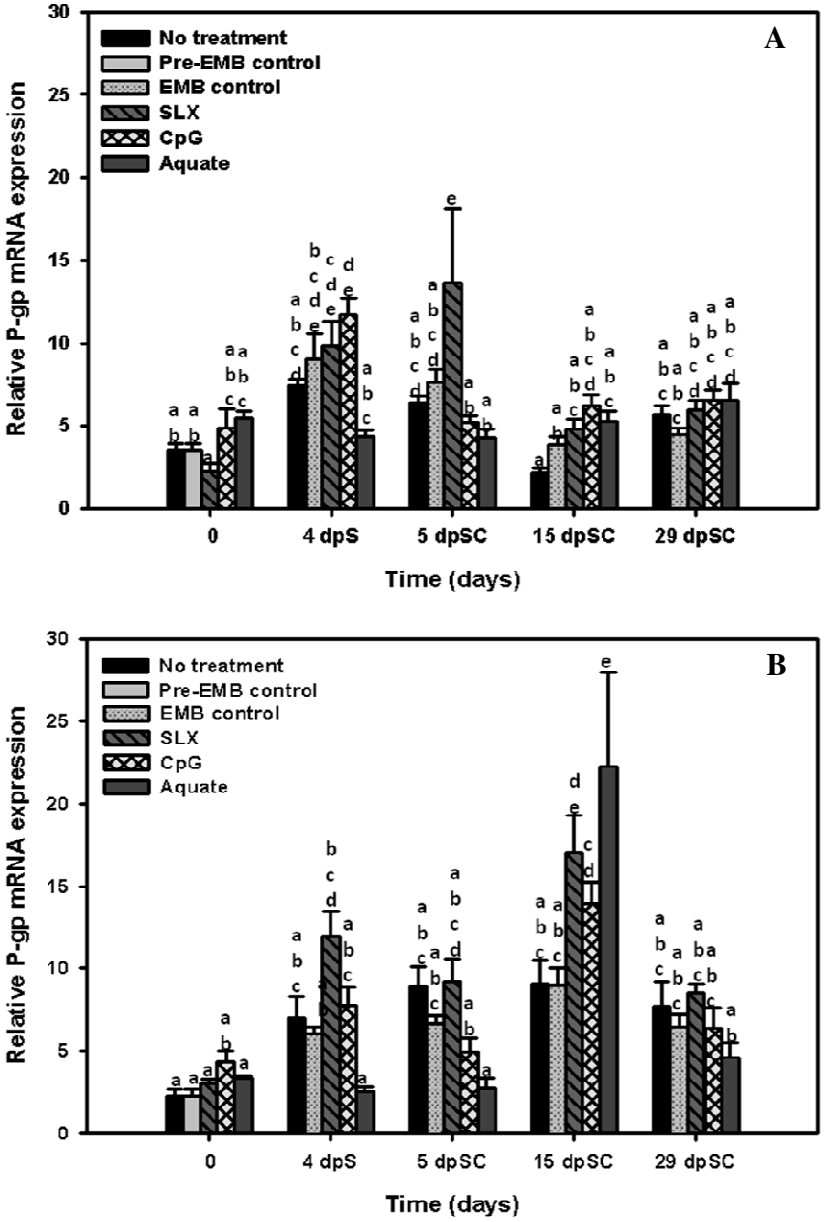

Figure 3.3. Relative P-glycoprotein mRNA expression in adult male (A) and female (B) *Lepeophtheirus salmonis* detached from *Salmo salar* fed first on different immunostimulant infused (SLX, CpG or Aquate®) diets for 7 weeks and then on 150 µg emamectin benzoate/kg fish biomass for 7 days (mean relative expression ± SEM) normalized to 3 reference genes - (glyceraldehyde-3-phosphate dehydrogenase, translation eukaryotic elongation factor 1α, structural ribosomal protein S20) and compared with a calibrator. *Lepeophtheirus salmonis* samples are first generation laboratory reared sea lice. Different superscripts (a, b, c, d, e) denote significant ($P < 0.05$) differences between two means (n = 6). No Tx = no immunostimulant treatment; Pre-EMB CTL = prior to EMB treatment/also not treated with immunostimulants; EMB CTL = EMB treatment only; dpS = days post-commencement of emamectin benzoate treatment; dpSC = days after cessation of EMB treatment.

3.5. Discussion

Boosting host immunity against invading pathogens and parasites through immunostimulation has been employed for disease management in aquaculture (reviewed by Ringo et al. 2011). However, the effects of prior host immunostimulation on chemotherapy are not well understood and may not always offer protection against a pathogen (Kunttu et al. 2009). The present study was aimed at determining the effects of prior host immunostimulation on EMB efficacy and mRNA expression of the putative EMB transporter, P-pg, in *L. salmonis*. In the present study, sea lice with different immunostimulant backgrounds were subjected to a 24 h EMB bioassay. Contrary to our prediction, sea lice associated with immunostimulant-fed salmon did not significantly differ with control values within each stage and sex category. However, EMB EC_{50} values for male sea lice increased >2-fold from preadult to adult stage for treatment and control groups while the female EC_{50} values remained unchanged and ~3 to 6-fold lower compared with the males at the adult stage. This is suggestive of stage and sex differences in EMB sensitivity, consistent with earlier studies (Westcott et al. 2008, Whyte et al. 2013).

Interaction between prior host immunostimulation and EMB efficacy was evident especially with sea lice % survival data (Table 3.4). Although immunostimulation was initially beneficial to the host salmon in rejecting infecting sea lice (18-19% of initial infection level) (Poley et al. 2013), it had the opposite effect upon subsequent EMB treatment 2 weeks after cessation of immunostimulant diet. Salmon with no immunostimulant treatment (EMB treated and untreated groups) had lower sea lice numbers compared with immunostimulant treated fish (Table 3.4) despite the fact that the different treatment groups had relatively similar EMB concentrations. Skin from EMB treated salmon had higher concentrations of EMB compared with the muscle in agreement with previous studies (Sevatdal et al. 2005, Whyte et al. 2011).

This is attributable to the lipophilic nature of EMB and is desirable for drug availability to sea lice through the mucus. While salmon muscle EMB concentration in the Aquate® group was relatively unchanged at 4 dpS and 5 dpSC, the concentration of the parasiticide for SLX group decreased from 4 dpS to 5 dpSC. Whether these differences are a result of differences in palatability (Whyte et al. 2011) of the different diets or the effects of the constituent immunostimulant is not known. Low levels of EMB, < 9 and < 17 ppb, were detected in the muscle and skin of the salmon that were not treated with EMB, a common occurrence with drug exposure in a recirculation system (pers. comm. Dr. S.K. Whyte, CAHS). A similar study that used a flow-through system reported low levels of EMB in the skin of untreated controls (Whyte et al. 2011). The results in this study are likely due to inherent inefficiency of the system to prevent trace levels of the parasiticide from entering EMB untreated tanks. Given that there was no measureable EMB in the control feeds (sensitivity > 15 $\mu g\ L^{-1}$) and these levels in the muscle and skin are so low, they can be considered as background values with no potential effects on observations.

Macrocyclic lactone resistance has been linked to changes in target site (Njue et al. 2004), increased metabolism and increased drug efflux (Wolstenholme & Kaplan 2012) but changes in structure or increased expression of the ATP-binding cassette (ABC) transporter, P-glycoprotein (*P-gp*), is widely believed to be the primary mechanism for loss of ML sensitivity (reviewed by Prichard & Roulet 2007). We have previously linked differences in EMB sensitivity to sex-based differences in mRNA expression of P-gp in sea lice (Chapter 2). Studies have also shown that *P-gp* plays a role in sex-based differences in drug efficacy and toxicity in humans (Sudchada et al. 2010), dogs and rats (Lespine et al. 2009), and parasitic nematodes (Prichard & Roulet 2007). In this study (Fig. 3.2 A and B), female sea lice had higher P-gp mRNA expression as

preadults compared with adults, male expression of the transporter was not significantly different at both preadult and adult stages, except for male sea lice from fish fed the Aquate® diet. Also, increases in the expression of P-gp occurred earlier in male compared with female sea lice. Timing of P-gp mRNA expression appears to be related to survival of the parasite during EMB treatment and may be the reason why male parasites had greater survival compared with females. Whether louse sex-based differences in EMB sensitivity are due to timing (present study), rather than level (Chapter 2) of P-gp mRNA expression, is unknown. While the study in Chapter 2 was performed using sea lice samples exposed to EMB *in vitro* in a 24 h bioassay, the current study employed sea lice samples previously exposed to the parasiticide while feeding on treated fish and over a longer period. These differences could explain why, in the earlier study, male sea lice expressed higher P-gp mRNA levels compared with female sea lice. A study to track timing of increasing P-gp expression in *L. salmonis* following on-host EMB treatment is in progress in our laboratory.

Differences in sea lice numbers observed among different treatment groups and controls (Table 3.4) can be linked to differences in P-gp expression especially for the female sea lice groups (Fig. 3.3B). Immunostimulant treatment groups that displayed >1.5 fold increase in P-gp mRNA expression recorded relatively higher % parasite survival. This was contrary to our hypothesis that prior host immunostimulation would cause higher sea lice mortality following subsequent EMB treatment compared with the administration of immunostimulants or EMB alone. Twenty-nine (29) dpSC was the most logical time point to use in assessing treatment outcome (% survival) but its choice could have influenced our conclusion. This is because the rate at which the sea lice numbers decreased over time was not linear, and in fact in some cases, sea lice numbers increased during the treatment thereby influencing final assessment of treatment

effects at 29 dpSC. For example, while % survival of male EMB treatment group at 5 dpSC was 17 % compared with 26.4 % at 29 dpSC; the values for the no EMB controls were 19.5 and 15.8 % at 5 and 29 dpSC, respectively.

The Aquate® diet contains yeast extracts, including β-glucans, potent PAMPs that boost the innate immune response through pro-inflammatory processes (Ringo et al. 2011). β-glucan decreased susceptibility of rainbow trout [*Oncorhynchus mykiss* (Walbaum)] to white spot disease [*Ichthyophthirius multifiliis* (Xueqin, Kania & Buchmann 2012)] through increased expression of major histocompatibility complex class II, C3 complement factor and lysozyme activity (De Baulny et al. 1996). However, some studies have associated β-glucan with exacerbation of infection intensity in *Artemia franciscana* (Kellogg), infected with *Vibrio campbelli* (Soltanian et al. 2007) and in juvenile rainbow trout (*O. mykiss*) treated for *Flavobacterium columnare* infection (Kunttu et al. 2009). In fact in the study by Kunttu et al. (2009), more mortalities were recorded for the *F. columnare* infected fish that received β-glucan than in infected immunostimulant untreated controls. In our study, the higher % survival in immuostimulant fed groups compared with controls following EMB treatment could be due to host tolerance to innate stimulation, immunosuppression and/or stress induced by prolonged (7 weeks) immunostimulant administration (Bricknell & Dalmo 2005). It has also been shown that increased plasma cortisol expression seen during stress from sea lice infection can lead to depressed immune responses (Johnson & Albright 1992). Although Roy et al. (2000) report no adverse effects of EMB administration up to 3.5 times the recommended dose (50 µg/kg fish biomass per day for 7 days), the 3 times dosage used in the field and in the current study may have immunosuppressive effects as well, as has been observed in ivermectin treatments in rabbits (Sajid et al. 2009). Immunosuppression of the hosts due to EMB may allow for greater survival

of sea lice in all EMB treatment groups when compared with untreated controls. Further research into interactions between EMB administration and host immunocompetence are required to answer these questions.

While SLX adult male sea lice had the same EMB EC_{50} values as the adult male EMB-untreated group, EMB EC_{50} values for the CpG male sea lice was ~1.6 fold lower compared with the two former (Table 3.3). Why sea lice from the adult male CpG group recorded lower EMB EC_{50} values compared with the rest of the adult male groups, including the no-immunostimulant background group, is unknown, but suggestive of an immunostimulant effect possibly on P-gp expression. Studies also indicate that P-gp expression can be affected by inflammatory responses whereby acute inflammation will cause increased expression of the transporter (reviewed by Ho & Piquette-Miller 2006), while the reverse is the case for chronic inflammatory responses (Llorente et al. 2000). The study by Llorente et al. (2000) linked *P-gp* over-activity in patients with rheumatic autoimmune disease to increased expression of TNFα.

The SLX, CpG, and Aquate® treated salmon had the highest number of sea lice and these same sea lice expressed higher levels of P-gp mRNA compared with the sea lice attached to salmon with no immunostimulant treatment. This suggests that administration of the immunostimulant feed for 7 weeks to the respective treatment groups could have induced chronic immune responses including inflammation and release of other immune factors and molecules within the sea louse. Since the occurrence of chronic inflammation was not investigated in the sea lice sampled, it could also be that chronic inflammation/wound healing in the host, as seen in previous studies of salmon infected with *L. salmonis* (Skugor et al. 2008) may have direct effects on sea lice P-gp mRNA expression. Recent work, however, has also shown that CpGs and yeast extracts administered through the feed can induce inflammation both

systemically and at the attachment site in Atlantic salmon infected with *L. salmonis* (Covello et al. 2011, Poley et al. 2013). As *P-gp* has been shown to mediate platelet activation factors and cytokine transport in human mesangial and T-cell populations, respectively (Drach et al. 1996, Ernest & Bello-Reuss 1999), it may serve the salmon louse in an immunomodulatory capacity into immunostimulated hosts.

Inflammatory processes and release of reactive oxygen/nitrogen species can induce oxidative/nitrosative stress, which may affect *L. salmonis* tissues in contact with the reaction/feeding site or within the salmon louse gut. The corollary to this would be cellular necrosis within *L. salmonis* foregut and potential depletion of ATP at the mitochondrial level. Enhanced production of *P-gp* within these affected sea louse tissues, in particular the gut, could provide enhanced protection to the parasite against stimulated host responses. Studies have shown that crustaceans possess β-glucan binding receptors and other PRRs responsive to different immunostimulants (Smith, Brown & Hauton 2003). The innate crustacean immune response has been exploited in disease control in kuruma shrimp, *Penaeus japonicus* (Bate) (Itami et al. 1998), and black tiger prawn, *P. monodon* (Fabricius) (Sritunyalucksana et al. 1999). Given the relationship between chronic innate immune response and P-gp expression (Llorente et al. 2000), it is possible that by-products of SLX, CpG, and Aquate® diet inflammatory induction interacted with the louse to induce over-expression of P-gp and consequently reduced EMB efficacy in the parasite. Perhaps less likely, the possibility remains that immunostimulants taken up by the host are only partially processed in the gut and/or systemically; and that some transportation of these molecules to the skin/mucosal membrane occurs, directly stimulating the parasite itself. Further investigation into the half-life and fate of these compounds within the host organism is needed to be able to verify these propositions more accurately.

Our study demonstrates that prior host Atlantic salmon immunostimulation may result in decreased EMB efficacy in sea lice, possibly due to over-expression of P-gp and/or induction of non-specific protective mechanisms within the parasite. This reinforces the importance and need for monitoring the effects of treatments, especially to avoid unintended consequences. Further studies are needed to elucidate the mechanisms by which host immunostimulation affects expression of ABC transporters and/or other mechanisms of resistance to parasiticides in *L. salmonis* and how immunostimulants potentially interact with ongoing treatment regimens.

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CHAPTER 4

SEA LICE POPULATION AND SEX DIFFERENCES IN P-GLYCOPROTEIN EXPRESSION AND EMAMECTIN BENZOATE RESISTANCE ON ATLANTIC SALMON FARMS IN THE BAY OF FUNDY, NEW BRUNSWICK, CANADA

4.1. Abstract

Parasitic sea lice, including *Lepeophtheirus salmonis* and *Caligus elongatus*, are a major challenge currently facing commercial salmon production. Attempts over several decades to control the parasite using drugs and chemicals have led to emergence of widespread drug resistant strains of the organisms. There are recent reports of reduced efficacy to emamectin benzoate (EMB), the major sea lice therapeutant within the last decade. However, there are fish farm locations in the Bay of Fundy, NB, Canada, where EMB-sensitive sea lice still exist. Previous studies have linked EMB resistance in sea lice to over-expression of the ATP-binding cassette (ABC) transporter, P-glycoprotein (P-gp) in the parasite. We investigated 1) whether reports of EMB treatment success in Grand Manan, Bay of Fundy, NB, can be explained through EMB bioassay and P-gp mRNA expression studies, 2) if other populations of sea lice not under EMB selective pressure possess similar EMB sensitivity as Grand Manan sea lice populations, and 3) the heritability of EMB sensitivity/resistance in *L. salmonis* populations. The EMB bioassay results indicated population, species, sex-based, and temporal differences in EMB EC_{50} values. *L. salmonis* collected from Grand Manan showed >2 fold lower EMB EC_{50} values compared with two reference populations, confirming the presence of relatively EMB-sensitive sea lice in Grand Manan. Sea lice reared in the laboratory maintained their EMB sensitivity status for up to three filial generations. *C. elongatus*, collected from Grand Manan also showed >2 fold lower EMB EC_{50} values compared with *L. salmonis* collected from the same site, suggesting species differences in EMB sensitivity in sea lice. Also addition of verapamil, a competitive inhibitor of *P-gp*, yielded no increase in *C. elongatus* EMB sensitivity. Taken together, these findings emphasize the need to understand and monitor for EMB resistance even in sea lice populations perceived to be EMB insensitive.

4.2. Introduction

Sea cage production of Atlantic salmon, *Salmo salar*, one of the most intensively farmed marine fish (Naylor & Burke 2005), is currently hampered by sea lice, ectoparasites of salmonids commonly found in marine environments. *Lepeophtheirus salmonis* and *Caligus elongatus* are the major species of sea lice that infect salmon in Atlantic Canada (Burka, Fast & Revie 2012). Strategies that have been used or are under development for sea lice control in salmon farms include biological predators (Deady, Varian & Fives 1995, Treasurer 2002), vaccines (Ross et al. 2006, Frost, Nilsen & Hamre 2007, Carpio et al. 2011), immunostimulation (Covello et al. 2012, Purcell et al. 2012), drugs and chemicals, as well as good management practices. The most effective intervention strategy has been chemicals or drugs administered either topically or in-feed. The macrocyclic lactones (MLs), which could be administered in-feed, to avoid the disadvantages (i.e. labor intensive, stress to fish, inability to simultaneously treat all cages on site, etc.) associated with bath applications, held promise as an efficacious treatment against all parasitic stages of sea lice (Smith & Clarke 1988, Roy et al. 2000). Ivermectin was the initial ML to be used in sea lice control (Smith & Clarke 1988), but the low therapeutic index discouraged its continued use (Johnson & Margolis 1993, Davies & Rodger 2000), hence, the development of another ML, emamectin benzoate (EMB; SLICE®), which had a better therapeutic index (Roy et al. 2000). Emamectin benzoate was initially effective for sea lice control at a dose of 50 µg kg^{-1} fish biomass for 7 days (Ramstad et al. 2002), but currently double and triple doses are used in some salmon farms in Atlantic Canada (M. Beattie, Department of Agriculture, Aquaculture and Fisheries of New Brunswick (NB), **http://www.dfo-mpo.gc.ca/science/enviro/aquaculture/rd2011/rdsealice-pou-eng.html**), as the manufacturer's recommended 50 µg kg^{-1} fish biomass for 7 days is no longer clinically

effective. Macrocyclic lactones interrupt neurotransmission in nematodes through the activation of glutamate-gated chloride channels, resulting in starvation, paralysis and, consequently, death of the parasite (Arena et al. 1995, Wolstenholme & Rogers 2005). Emamectin benzoate has high efficacy against all parasitic stages of sea lice, disrupting the life cycle at several points (Stone et al. 1999). Lipophilicity of EMB creates sustained effective tissue concentrations leading to protection for prolonged periods (Horsberg 2012). These attributes quickly made EMB the drug of choice for sea lice control on fish farms in the Bay of Fundy, NB (Westcott, Hammell & Burka 2004). However, use of EMB has declined due to resistance development, a consequence of over-reliance on the drug for over a decade. Resistance to EMB by *L. salmonis* has been reported in Atlantic Canada and Europe (Hjelmervik et al. 2010, Westcott et al. 2010) and in *C. rogercresseyi* in Chile (Bravo, Sevatdal & Horsberg 2008).

While resistance to EMB by multiple species of sea lice is indisputable, some refuges for the parasite remain as not all sea lice populations within a given area are resistant to the drug (Westcott et al. 2010). There are reports that sea lice populations on Atlantic salmon farms in Grand Manan, an island in the Bay of Fundy, approximately 30 km from the mainland, are more EMB-sensitive compared with sea lice populations at locations close to the mainland (Jones et al. 2012). Also, within a given *L. salmonis* population, males have been shown to be more EMB-resistant compared with females (Chapter 2, Westcott et al. 2008, Heumann et al. 2012, Whyte et al. 2013), but sex differences in EMB sensitivity have not been explained. Such knowledge could be exploited in tracking EMB resistance development in salmon farms more efficiently.

The narrow spectra of treatment options and ability of sea lice to develop drug resistance underpin the need to understand how these parasites respond to drugs used for their control. Macrocyclic lactone resistance in arthropods and nematodes has been linked to changes in target

site (Njue et al. 2004), but changes in structure or increased expression of the ATP-binding cassette (ABC) transporter, P-glycoprotein (*P-gp*), is widely believed to be the primary mechanism for loss of ML sensitivity in nematodes (reviewed by Prichard & Roulet 2007). Results of Chapter 2 demonstrated a temporal increase in P-gp mRNA expression in *L. salmonis* samples collected from the Bay of Fundy from 2002 to 2011. Also, studies by Heumann et al. (2012) and findings from Chapter 2 showed that sub-acute EMB exposure induces over-expression of P-gp mRNA in *L. salmonis*. Competitive inhibition of *P-gp* in *L. salmonis* using verapamil caused increased EMB sensitivity in the parasite, suggesting that *P-gp* plays a role in sea lice resistance to the drug (Chapter 2).

The objectives of this study were 1) to identify whether reports of EMB treatment success in Atlantic salmon farms in Grand Manan can be explained through EMB bioassay and P-gp mRNA expression studies, 2) to determine if other populations of sea lice not under EMB selective pressure display a similar or differential response to EMB bioassay assessment as sea lice samples collected from Grand Manan, and 3) whether any differences identified between *L. salmonis* populations are heritable and result in differences in EMB sensitivities following drug exposure on or off the host.

4.3. Materials and methods

4.3.1. Chemicals

Emamectin benzoate (PESTANAL®) and chemicals used for this study were of analytical grade and purchased from Sigma-Aldrich, St. Louis, MO, except where stated otherwise.

4.3.2. *Salmo salar* population

All Atlantic salmon used for this study were procured from Buckman's Creek Hatchery Ltd., Pennfield, NB, and transported to the Atlantic Veterinary College (AVC) Aquatic Facility. The fish were weighed (97 ± 8.3 g) and randomly allocated to tanks at 20 fish per tank (330 L) and allowed to acclimatize to the flow-through system for 3 weeks. The system was then changed to recirculation and the salinity gradually raised to 31-33 g L^{-1} with Instant Ocean® (Aquarium Systems, Cincinnati, OH) over 7 days and allowed to acclimatize again for 3 weeks. The fish were fed 1% body weight base feed (Northeast Nutrition Ltd., Truro, NS), as 2.5 mm^3 pellets and maintained on a 14-h light/10-h dark cycle. Water temperature (11 ± 1°C), concentration of ammonia, nitrite, oxygen and nitrate in the water were closely monitored and maintained at optimal levels.

4.3.3. *Lepeophtheirus salmonis* and *Caligus elongatus* populations

According to Chang et al. (2007), Atlantic salmon farms in southwestern New Brunswick are grouped into six Bay Management Areas [BMAs; (Fig. 4.1)]. BMA 1 is made up of Passamaquoddy Bay, Deer Island, and a large part of Campobello Island; BMA 2 consists of 2a (Back Bay, Bliss Harbour, and Lime Kiln Bay) and 2b (eastern Grand Manan Island and White Head Island); BMA 3 also consists of 3a (Beaver Harbour to Haleys Cove) and 3b (southern Grand Manan); BMA 4 (Harbour de Loutre, Campobello Island); BMA 5 (Dark Harbour, Grand Manan Island) and BMA 6 (Letete Passage). The present study was carried out using sea lice samples collected from BMAs 1, 2a and 2b. While sea lice population from BMAs 1 and 2a are believed to be EMB resistant, BMA 2b sea lice samples are suspected to be EMB sensitive (Jones et al. 2012).

Adult male and female *L. salmonis* were carefully removed from host Atlantic salmon from fish farms in BMA 1 and BMA 2a, Bay of Fundy, NB. The samples were brought back alive to the laboratory in cold (10°C) sampling site seawater and stored in a cold incubator at 10°C overnight with aeration. The samples were collected from EMB-resistant populations of *L. salmonis* based on treatment efficacy in the area [(Jones et al. 2012) and confirmed through previous EMB bioassays] and will be referred to as R0-1 (collected in October 2011) and R0-2a (collected in March 2012) in this study, for BMA 1 and BMA 2a, respectively. Adult male and female *L. salmonis* samples were previously collected in November 2011 from another location within BMA 2a and will be referred to as R0a-2a. Approximately 14 to 18 wks after the October 2011 sea lice collection, 2 batches (4 weeks apart) of adult male and female *C. elongatus* were collected from BMA 1 (the same salmon farm location as R0-1) and brought back to the laboratory in a similar manner. Samples of *L. salmonis* were collected alongside the first *C. elongatus* sampling for comparison purposes. Adult male and female *L. salmonis* were also collected (April 2012) in the same manner as above from Grand Manan (BMA 2b). The Grand Manan *L. salmonis* population has been reported to be more EMB sensitive compared with *L. salmonis* from other sites within the Bay of Fundy (Jones et al. 2012) and will be referred to as S0-2b. Finally, archived adult female *L. salmonis* collected from Grand Manan (BMA 2b) in 2005 and stored at -80°C were used to verify changes in the expression of P-gp mRNA over time compared with samples collected from the same location in 2012.

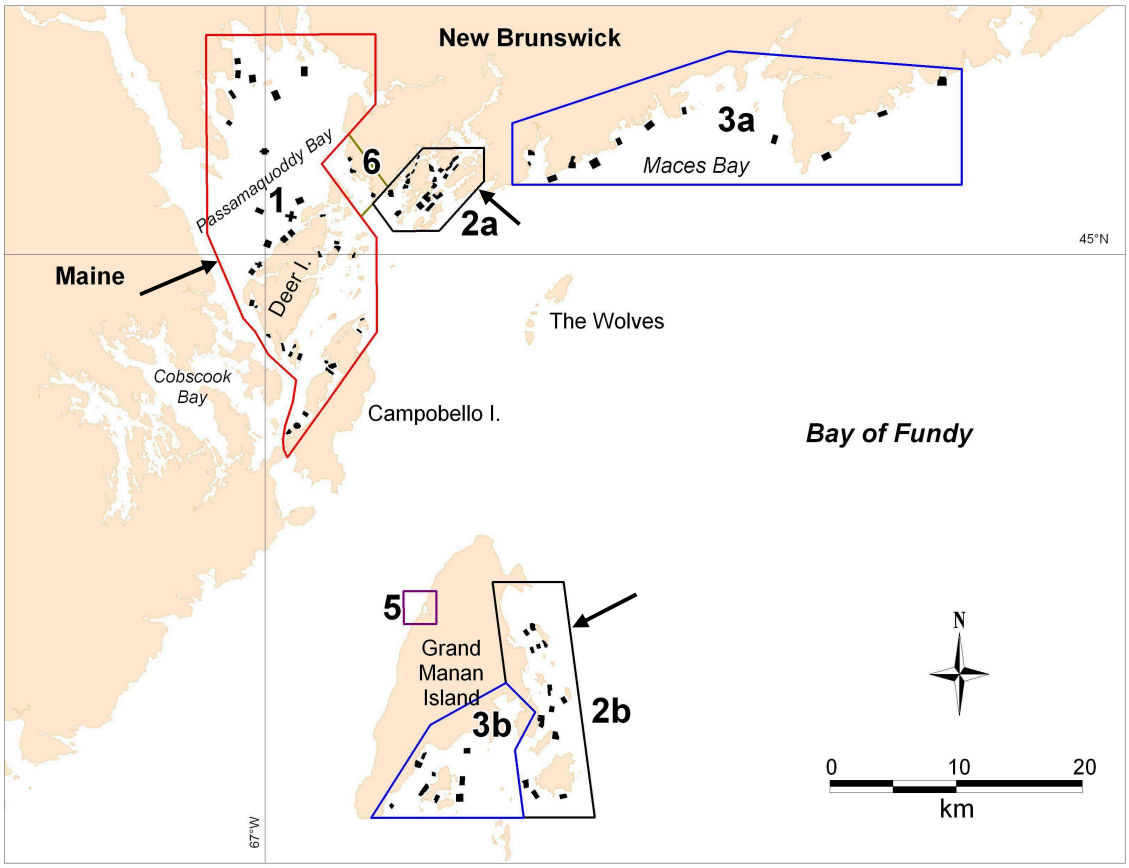

Figure 4.1. Map of Southern New Brunswick showing distribution of Atlantic salmon farms (black spots) within the different Bay Management Areas (1, 2a, 2b, 3a, 3b, 5 and 6). Courtesy of Dr. Blythe Chang, Department of Fisheries and Oceans Canada, St. Andrews Biological Station, St. Andrews, NB, Canada. Arrows point at zones/areas of sea lice collection.

4.3.4. Responses to EMB exposure across sea lice populations

4.3.4.1. EMB bioassay

A total of 5 EMB bioassays were carried out in this study to compare EMB sensitivity of sea lice populations collected from 3 different BMAs (1, 2a and 2b), as well as their laboratory reared progeny. All the bioassays were carried out at 10°C for 24 h using adult stages of both sexes according to methods and criteria described in Chapter 2. Brief descriptions of the bioassays are as follows:

Bioassay I: EMB EC_{50} values determined for in-house reared F1 generation (R1-1) of *L. salmonis* collected from BMA 1 (R0-1) using 0, 10, 100, 300 and 1000 ppb EMB concentrations.

Bioassay IIa: EMB EC_{50} values determined for *L. salmonis* collected from BMA 2a (R0a-2a) at 0, 100, 200, 400 and 1000 ppb EMB concentrations.

Bioassay IIb: EMB EC_{50} values determined for in-house reared F1 generation (R1-2a) of *L. salmonis* collected from BMA 2a (R0-2a) and F0 *L. salmonis* collected from BMA 2b (S0-2b) using 0, 0.1, 25, 300 and 1000 ppb EMB concentrations. The choice of F1 and F0 generations of R1-2a and S0-2b, respectively, were a combination of logistical (i.e. R1-2a were already one generation ahead of S0-2b) and experimental reasons (i.e. to ascertain how different R1-2a are from S01-2b in EMB susceptibility given the generational differences).

Bioassay III: EMB EC_{50} values determined for *C. elongatus* collected from BMA 1 using 0, 30, 100, 300 and 1000 ppb EMB concentrations.

Bioassay IV: EMB EC_{50} values determined for in-house reared F2 generation of *L. salmonis* collected from BMA 2a (R2-2a) and in-house reared F1 generation of *L. salmonis* collected from BMA 2b (S1-2b) using 0, 0.1, 25, 300 and 1000 ppb EMB concentrations.

The differences in concentration of EMB among the bioassays are based on suspected variations in the parasiticide sensitivity of the test sea lice populations.

4.3.4.2. RNA extraction

Total RNA was extracted from Bioassay IIb survivors and the archived samples following standard procedures as described in Chapter 3. Bioassay IIb survivors were chosen based on suspected population-based differences in EMB sensitivity between R1-2a and S0-2b samples.

Briefly, one (female) or two (male) [to obtain optimal RNA yield for males as the females are twice bigger than the males] *L. salmonis* were homogenized in a 5 mL plastic tube containing 0.8 mL trizol reagent using an electric tissue homogenizer (VWR, Mississauga, ON). The RNA pellets derived following standard washes and precipitation were re-suspended in molecular grade water. A subset of RNA samples was verified for quality using Experion™ RNA StdSens Chips (Bio-Rad Laboratories, Hercules, CA). Cutoff for good quality RNA was ≥ 8 on the RNA integrity scale. RNA concentration and 260/280 nm ratio were determined using a Nanodrop Spectrophotometer 2000 (Thermo Scientific, Wilmington, DE). The samples were then stored at -80°C prior to further use.

4.3.4.3. Reverse transcription quantitative PCR

RT-qPCR steps were conducted according to standard procedures and have been described in Chapter 3. Briefly, DNA contamination was eliminated from the RNA samples (Bioassay IIb survivors) using TURBO DNase-*free*™ kit (Ambion, Carlsbad, CA). One microgram of each DNA-free RNA sample was reverse transcribed using Reverse Transcription System (Promega, Madison, WI), according to manufacturer's instructions. Successful

elimination of DNA contamination was confirmed by performing qPCR using No Reverse Transcriptase-treated RNA control samples. The qPCR reactions were done using GoTaq® qPCR Master mix (Promega) on a Realplex thermocycler (Eppendorf, Mississauga, ON) and the cycling conditions were as reported previously (Chapter 3). Relative P-gp mRNA levels were normalized using 4 reference genes employed in a previous study (Chapter 2) -glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, translation eukaryotic elongation factor 1α (eEF1α), and structural ribosomal protein S20 (RPS20) - using geNorm software (Vandesompele et al. 2002). Due to its relatively high abundance and instability 18S rRNA was excluded from generation of the normalization factor during geNorm analyses. The primers for the different genes were the same as reported in Chapter 2. Reaction efficiency (%) of the primer sets were 103 (P-gp), 98 (eEF1α), 91 (18S rRNA), 100 (RPS20) 99 (GAPDH). Relative fold differences in P-gp mRNA level were determined using the $2^{-\Delta\Delta Cq}$ method normalized to the calibrator.

4.3.4.4. P-glycoprotein inhibition test

P-glycoprotein inhibition test was conducted according to the method described in Chapter 2 using adult female *C. elongatus* collected from BMA 1. Briefly, sea lice samples were subjected to EMB bioassay at 10°C for 24 h using 0, 30, 100, 300 and 1000 ppb with or without 10 µM verapamil, a *P-gp* inhibitor. The choice of 10 µM verapamil was based on results of previous studies (Chapter 2).

4.3.4.5. On-host comparison of *L. salmonis* populations following triple dose EMB treatment: series I

Having confirmed susceptibility of *L. salmonis* from BMAs 2a and 2b (Bioassay IIb), we determined whether EMB resistance phenotype would be passed on to the next generation of *L.*

salmonis grown in-house (Table 4.1). F1 generation of *L. salmonis* from BMA-2b (S1-2b) was reared, at the same time on Atlantic salmon (243.5 ± 10.3 g) in different 330 L tanks, as a confirmed F2 resistant population (R2-2a) from BMA 2a. Upon reaching the preadult stage, male and female *L. salmonis* from both groups were again tested for EMB sensitivity (Bioassay IV; sub-section 4.3.4.1). These two *L. salmonis* populations were exposed to EMB on-host whereby, following removal of sea lice for EMB Bioassay IV, two tanks of fish infected with R2-2a and S1-2b were placed on 7 day triple-dose EMB treatment (150 μg kg^{-1} fish biomass; actual EMB concentration in feed was 120 mg L^{-1} based on the methods described in Chapter 2).

Briefly, *L. salmonis* egg strings harvested from BMA 2a were hatched in-house, yielding F1 generation (R1-2a) nauplii, and allowed to develop to the copepodid life stage in static seawater incubators prior to exposure to naïve Atlantic salmon (Chapter 3, Covello et al. 2012). *Lepeoptheirus salmonis* were reared to adult stage on their hosts and allowed to in-breed to produce egg strings for a second filial generation: R2-2a. S0-2b egg strings were also hatched in the lab and the resultant copepodids [S1-2b (120 copepodids/fish)], as well as R2-2a (50 copepodids/fish), were used to infect salmon separately. Due to time constraints on egg extrusion, S1-2b copepodids were used to infect Atlantic salmon (1 tank) 8 days prior to 2 tanks of a separate group of salmon in a different system being infected with R2-2a copepodids. Again, the choice of F1 and F2 generations of S1-2b and R2-2a, respectively, were a combination of logistical (i.e. R2-2a were already one generation ahead of S1-2b) and experimental reasons (i.e. ascertain how different R2-2a will be from S1-2b in EMB susceptibility despite generational differences). Prior to infections, one-way water flow valves were installed in the recirculation system to prevent backpressure of water and exposure of *L.*

salmonis to the biofilter. To ensure maintenance of single cultures in each system, 100 µm meshes were also placed over inflow valves to prevent cross-infection of sea lice.

Upon >90% of the *L. salmonis* reaching preadult life stages, six fish per tank were euthanized using 250 mg L^{-1} MS-222 (Syndel Laboratories Ltd) and the sea lice enumerated and collected. One day later, the 7 day triple-dose EMB treatment began for two tanks (S1-2b and R2-2a), while the third tank (R2-2a) was maintained on non-medicated feed (i.e. no EMB treatment). Sea lice were then collected at two time points as follows: One day post-commencement (1 dpS) of the 7 day EMB treatment, fish (n=5) were euthanized for each treatment group and *L. salmonis* samples collected, staged, and enumerated. This was repeated 13 days post-cessation (dpSC) of the 7 day EMB treatment, with the exception that 10 fish per group were sampled on this day from each of the treatment tanks; 20 fish were also sampled from the untreated tank. All *L. salmonis* samples collected were flash-frozen on dry ice within 2 min of removal from the tank and stored at -80°C for future genetic analysis.

4.3.5. Responses to EMB exposure of *L. salmonis* from susceptible and resistant crosses

4.3.5.1. Crossbreeding of EMB-sensitive and -resistant *L. salmonis*

To assess whether EMB sensitivity/resistance was heritable and affected by different parental backgrounds, we performed several crosses using EMB-resistant and -sensitive *L. salmonis* samples collected from the same areas described above: BMA 2a and BMA 2b, respectively (Table 4.2). R2-2a males were mated with S1-2b females at 1:11 (male to female ratio) on single fish per tank as follows: Four (4) fish with S1-2b preadult/adult male and preadult female *L. salmonis* were anesthetized [250 mg L^{-1} MS-222 (Syndel Laboratories Ltd)] individually. While the males had begun to exhibit mate guarding, the pairs were pre-copulatory

and, as such, all females were virgins at this stage. All male *L. salmonis* were removed leaving only preadult females on the fish. Then, as the fish recovered in individual recovery baths, single adult male R2-2a were added to each recovery bath and inspected to ensure parasite attachment to the host. If the male R2-2a failed to attach within 10 sec, it was replaced with a new male R2-2a. On full recovery, each fish was returned to a 200 L housing tank alone and maintained for the remainder of the mating experiment.

Three weeks post initiation of mating the fish were inspected to ensure sea lice remained attached on the fish. The inspection was repeated 6 weeks post initiation of mating during which males from the three successful mating groups were removed and flash-frozen for future genetic analysis. One of the 4 fish lost all the female *L. salmonis* leaving only the male parasite; hence, this fish was removed from further study. The first batch of egg strings (~44) collected from the successful crosses had <10% develop to the copepodid stage. Twelve days later, ~40 egg strings were collected (i.e. 2nd batch of egg strings) and successfully hatched and developed to copepodids [i.e. cross-1 (RX1S F1) ~350 copepodids from 9 egg strings; cross-2 (RX2S F1) ~1000 copepodids from 16 egg strings; cross-3 (RX3S F1) ~400 copepodids from 14 egg strings; R = EMB resistant; S = EMB sensitive; X = crossbred; 1, 2 and 3 = group number; F1 = first filial generation].

Table 4.1. Summary of experimental outline for on-host (Atlantic salmon) comparison of *Lepeophtheirus salmonis* populations following triple dose (150 µg/kg fish biomass) emamectin benzoate (EMB) treatment.

Steps	1	2	3	4	5 (1 dpS)	6 (13 dpSC)
Activity	Egg strings collected from BMA 2a hatched to yield R1-2a	R1-2a in-bred in the laborartory yielding R2-2a; egg strings collected from BMA 2b (S0-2b) hatched to yield S1-2b	Atlantic salmon (fish) infected with R2-2a and S1-2b	On reaching preadult stages, R1-2a and S1-2b infected fish placed on 7 days EMB treatment at 150 µg/kg fish biomass	Fish sampled for sea lice infection level and staging	Fish sampled for sea lice infection level and staging

BMA = Bay Management Area, Bay of Fundy, NB, Canada; R1-2a = F1 generation laboratory reared *L. salmonis* hatched from F0 sea lice egg strings collected from BMA 2a; S0-2b and S1-2b = F0 (field) and F1 (laboratory reared), respectively, generation *L. salmonis* collected from BMA 2b; dpS = days post-commencement of EMB treatment; dpSC = days after cessation of EMB treatment.

Table 4.2. Summary of experimental outline for on-host (Atlantic salmon) comparison of *Lepeophtheirus salmonis* crosses following triple dose (150 µg/kg fish biomass) emamectin benzoate (EMB) treatment.

Steps	1	2	3	4	5	6 (treatment day 0)	7 (7 dpS)
Activity	R2-2a males mated with S1-2b females (1:11, male to female ratio) on host Atlantic salmon (fish)	Fish inspected 3 weeks post initiation of mating	Fish inspection repeated 6 weeks post initiation of mating	8 weeks post initiation of mating, egg strings hatched to copepodids: RX1S F1, RX2S F1 and RX3S F1. RX1S F1 and RX3S F1 combined and referred to as RX1-3S F1	Fish infected with RX2S F1, RX1-3S F1 and R3-2a	At preadult stage, infected groups of fish (RX2S F1, RX1-3S F1 and R3-2a) were sampled for sea lice infection level and placed on 7 days EMB treatment at 150 µg/kg fish biomass	Fish sampled for sea lice collection, infection level and staging

R2-2a = F2 generation laboratory reared *L. salmonis* derived from F0 sea lice egg strings collected from Bay Management Area (BMA) 2a, Bay of Fundy, NB, Canada; S1-2b = F1 generation laboratory reared *L. salmonis* hatched from F0 sea lice egg strings collected from BMA 2b; dpS = days post-commencement of EMB treatment; R = EMB resistant; S = EMB sensitive.

4.3.5.2. Single dose EMB bioassay

A single bioassay was conducted in this sub-study, as described above with slight modifications; single dose of EMB (200 ppb) was used (Westcott et al. 2010). A brief overview of the bioassay is as follows:

Bioassay V: EMB sensitivity was determined for preadult male and female RX2S F1, RX3S F1, and R3-2a (i.e. progeny from R2-2a; see sub-section 4.3.5.3 below) collected prior to commencement of the second EMB on-host triple dose treatment.

4.3.5.3. On-host comparison of *L. salmonis* populations following triple dose EMB treatment: series II

As in the first on-host EMB exposure, the copepodid life stages were cultivated in static seawater incubators prior to exposure to naïve (n=15/tank) Atlantic salmon (Chapter 3, Covello et al., 2012). RX2S F1 copepodids (n~1000) were used to infect one tank of salmon (~70 sea lice/salmon); RX1S F1 and RX3S F1 copepodids were combined (will be referred to as RX1-3S F1; n~750) and used to infect a second individual tank of salmon (~50 sea lice/salmon); and F3 generation of *L. salmonis* collected from BMA 2a [(R3-2a) (~2350 copepodids)] generated at the same time were used to infect two tanks of salmon (~70 sea lice/salmon). Despite synchronized hatching of egg strings, RX1S F1, RX2S F1 and RX3S F1 all developed to copepodids slower and, as such, were used to infect salmon 2 days later than fish infected with R3-2a copepodids. Again, upon >90% of the sea lice reaching preadult life stages, three fish per tank were euthanized and the parasites enumerated and collected from each tank. A triple dose EMB treatment (150 μg kg^{-1} fish biomass) was administered to 3 tanks (RX2S F1, RX1-3S F1, R3-2a), with another tank (R3-2a) on control feed as described previously. Fish were euthanized and the

sea lice counted and collected from the tanks 7 days post cessation of EMB treatment. As with the first on-host EMB exposure, all *L. salmonis* samples collected were frozen on dry ice within 2 min of removal from the tank and stored at -80°C for future genetic studies.

4.3.6. Statistical analysis

The half-maximal effective concentration (EC_{50}) for EMB was derived using US Environmental Protection Agency Trimmed Spearman-Kaber (TSK) software version 1.5 (Hamilton, Russo & Thurston 1977). Differences in relative P-gp mRNA expression were determined by performing multivariate analysis of variance (MANOVA) using STATISTICA statistical software (StatSoft Inc., Tulsa, OK). Least significant difference post-hoc analysis was also conducted using STATISTICA to determine significant differences in P-gp mRNA expression among the different samples. Statistical significance was set at $p < 0.05$ and/or ≥ 1.5 fold changes in relative expression. Graphs of relative P-gp mRNA expression were plotted using SigmaPlot 10.0 (Systat Software Inc., Chicago, IL).

4.4. Results

4.4.1. Responses to EMB exposure across sea lice populations

In-house reared F1 generation adult *L. salmonis* from BMA 1 (R1-1) showed no significant difference in EC_{50} values in both sexes of the parasite (Table 4.3, Bioassay I). Similar EC_{50} values were also observed in BMA 2a field and F1 generation (R0a-2a and R1-2a, respectively) adult male *L. salmonis* while the females exhibited lower EC_{50} values (Table 4.3; Bioassay IIa and IIb). S0-2b, collected from Grand Manan Island (Fig. 4.1) and suspected of greater susceptibility to EMB, recorded EMB EC_{50} values lower in both sexes with 63 ppb for the adult male and 75 ppb for the adult female compared with in-house reared F1 generation of

L. salmonis collected from BMA 2a [R1-2a (Table 4.3; Bioassay IIb)]. Another comparison was made using a different species of sea lice, *C. elongatus*. Adult female *C. elongatus* and *L. salmonis* were collected simultaneously from BMA 1, the same source as the *L. salmonis* used for Bioassay I (Table 4.3). Although the number of *L. salmonis* collected was insufficient to determine EMB EC_{50} values, all the *L. salmonis* survived EMB exposure at ≤ 100 ppb, in agreement with the R1-1 bioassay result. *Caligus elongatus*, however, exhibited lower EC_{50} values (Bioassay III) compared with R1-1 EC_{50} values, i.e. in the same range as the suspected susceptible population of *L. salmonis* from Grand Manan (Bioassay IIb; Table 4.3).

4.4.2. Reverse transcription quantitative PCR

To determine if *P-gp* was involved in differential resistance to EMB across sea lice populations, RT-qPCR was used to analyze P-gp mRNA expression in archived *L. salmonis* from BMA 2b, as well as, Bioassay IIb survivors. There were no significant differences in P-gp mRNA expression in adult female *L. salmonis* collected from BMA 2b in 2005 and 2012 (Fig. 4.2). It would have been ideal to verify temporal differences in relative P-gp mRNA expression for the male *L. salmonis*, but, unfortunately, only female samples were archived.

P-glycoprotein mRNA expression, however, differed between BMAs 2a and 2b *L. salmonis* (R1-2a and S0-2b, respectively) within Bioassay IIb survivors. Relative P-gp mRNA expression for the EMB-untreated male R1-2a (0 ppb) group was significantly higher than male and female S0-2b groups as well as the female R1-2a no treatment groups (Fig. 4.3, $P < 0.05$).

The transporter was induced more readily in resistant male *L. salmonis* (R1-2a) compared with the females and the relatively less EMB sensitive (S0-2b) male populations. Increasing concentrations of EMB caused significant differences in relative P-gp mRNA expression between male R1-2a and S0-2b sea lice samples, but not in their female counterparts. Male R1-

2a 300 and 1000 ppb EMB treatment groups displayed significantly higher relative P-gp mRNA expression compared with the other treatment groups ($P < 0.05$).

4.4.3. P-glycoprotein inhibition test

As there was no sequence information for *C. elongatus* P-gp at the time of this study, we were unable to directly assess P-gp mRNA expression pattern in BMA 1 *C. elongatus*. However, we tested the susceptibility of adult female *C. elongatus* to EMB in the presence of a *P-gp* inhibitor, verapamil. Exposure of adult female *C. elongatus* to increasing concentrations of EMB with or without 10 µM verapamil yielded EMB EC_{50} values (confidence limits) of 160 (140, 182) and 72 (55, 95) ppb, respectively.

4.4.4. On-host exposure of *L. salmonis* populations to triple dose EMB

The first on-host exposure of R2-2a and S1-2b to EMB revealed higher % survival of R2-2a compared with S1-2b (Table 4.4). A single tank of fish infected with R2-2a maintained without EMB treatment showed similar mortality over the course of the experiment as EMB on-host treated R2-2a [22.8 ± 1.2 (mean ± SEM) sea lice per fish at day 0, and 9.4 ± 1.0 sea lice per fish at 13 dpSC]. Unfortunately due to low sea lice numbers, we were unable to maintain an untreated S1-2b infection group. Also, standard error of mean for S1-2b at day 0 was not obtainable as only total lice were counted over the six fish and not per individual fish.

A second on-host EMB exposure was conducted to compare the sensitivity of progeny from crosses-1 and -3 (RX1-3S F1), cross-2 (RX2S F1) and R3-2a, an in-bred progeny of R2-2a. The crosses recorded proportionately higher sea lice reductions from day 0 to 7 compared with the EMB-treated and untreated R3-2a groups (Table 4.5).

Despite infection within 2 days of each other the crosses were significantly delayed in terms of stages by the end of the trial. RX1-3S F1 comprised of 75% adult male and 25% preadult females while RX2S F1 was 33% adult male and 67% preadult female. The two R3-2a tanks were 60% adult males, 37% adult females and 3% preadult females.

4.4.5. Response of *L. salmonis* from susceptible and resistant crosses to single dose EMB exposure

While male and female R3-2a survived exposure to 200 ppb EMB (Bioassay V; Table 4.3), lower % survival was observed in sea lice, especially females, from the two crosses (RX1-3S F1 and RX2S F1).

Table 4.3. Sensitivity of adult male and female sea lice to emamectin benzoate (EMB) in a 24 h bioassay.

Bioassay identity	Sea lice source (and identity)	Emamectin benzoate concentrations (ppb)	Results [(EC_{50}- ppb; 95% confidence interval) or (% Survival)]
I	BMA 1 (R1-1)	0, 10, 100, 300, 1000	EC_{50}: Male- 329 (275, 394) *Female- 304 (241, 383)
IIa	BMA 2a (R0a-2a)	0, 100, 200, 400, 1000	EC_{50}: Male- 840 (614, 1047) Female- 254 (218, 296)
IIb	BMA 2a (R1-2a) BMA 2b (S0-2b)	0, 0.1, 25, 300, 1000	EC_{50}: R1-2a: Male- 403 (230, 706) Female- 170 (56, 519) S0-2b: Male- 63 (11, 352) Female- 75 (13, 432)
III	BMA 1 *C. elongatus*	0, 30, 100, 300, 1000	EC_{50}: Male- 105 (33, 334) Female- 55 (43, 69)
IV	BMA 2a (R2-2a) BMA 2b (S1-2b)	0, 0.1, 25, 300, 1000	EC_{50}: R2-2a: Male- 403 (230,706) Female- 218 (87, 548) S1-2b: Male- 421 (258, 686) Female- 274 (146, 516)
V	BMA 2a (R3-2a) Crosses (RX1-3S F1) (RX2S F1)	200	% Survival: *R3-2a : Male- 100; Female- 100 *RX1-3S F1: Male- 94; Female- 70 *RX2S F1: Male- 100; Female- 67

All sea lice samples are *Lepeophtheirus salmonis* except where stated otherwise and were collected from the Bay of Fundy, NB, Canada. The differences in concentration of EMB are based on suspected variations in sensitivity of the test sea lice populations to the parasiticide. All sea lice samples are adult stage except where indicated otherwise: *, preadult stage. % control mortality was ≤ 5% for all the bioassays.

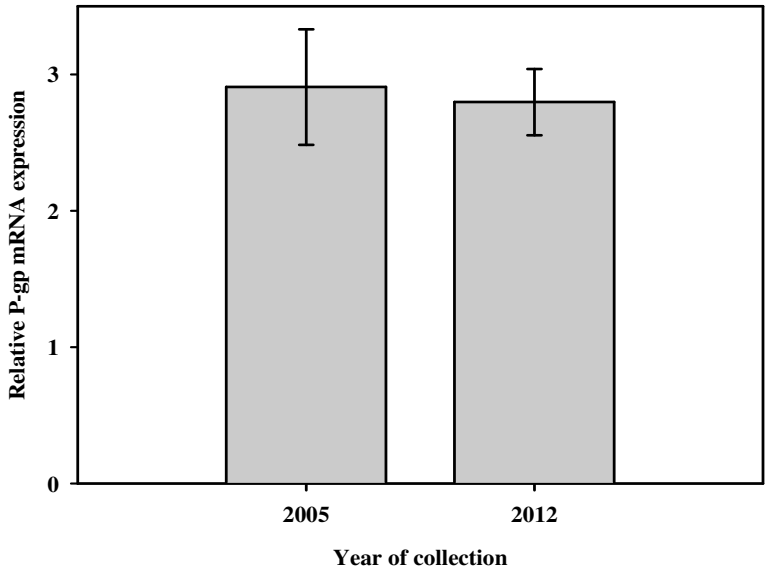

Figure 4.2. Relative P-glycoprotein mRNA expression (mean relative expression ± SEM; n = 5) in adult female *Lepeophtheirus salmonis* (sea lice) collected in 2005 (March and May) and April 2012 from Atlantic salmon farms in Grand Manan (Bay Management Area 2b), Bay of Fundy, NB, Canada.

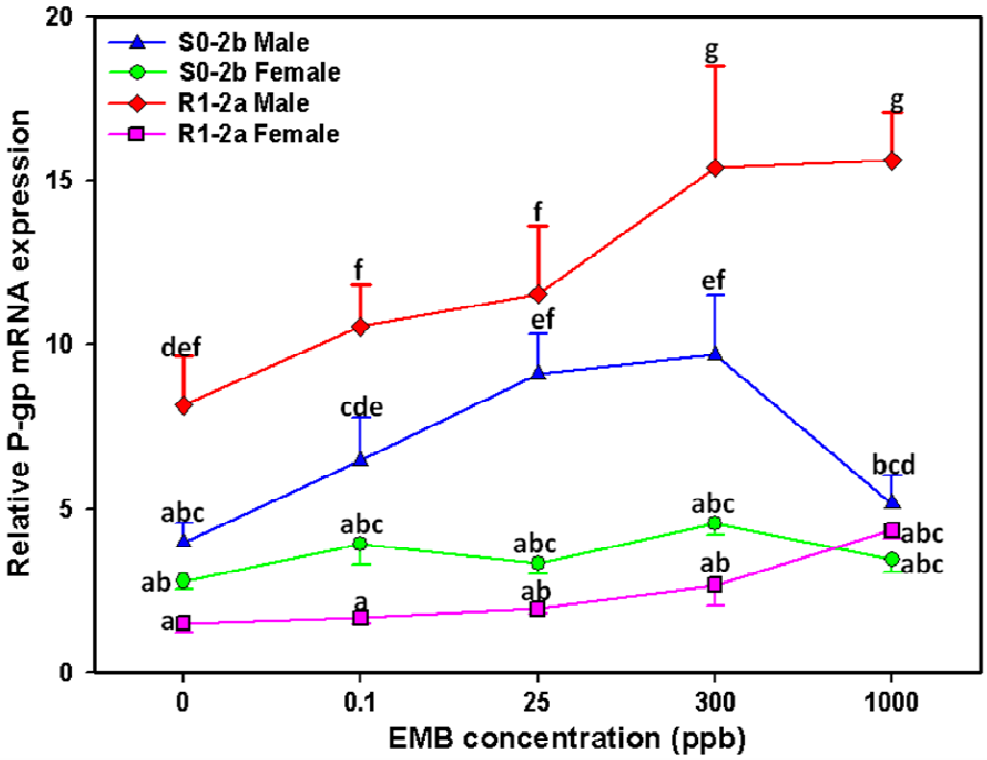

Figure 4.3. Relative P-glycoprotein mRNA expression (mean relative expression ± SEM) in adult male and female *Lepeophtheirus salmonis* emamectin benzoate bioassay (EMB) survivors. *Lepeophtheirus salmonis* samples were collected in 2012 from Atlantic salmon farm locations at 2 different Bay Management Areas (R1-2a = EMB resistant laboratory-reared F1 sea lice from BMA 2a; S0-2b = EMB sensitive field sea lice from BMA 2b) in the Bay of Fundy, NB, Canada. Different superscripts (a, b, c, d, e, f, g) denote significant ($p<0.05$ and/or ≥ 1.5 fold) difference between two means (n = 5).

Table 4.4. Sensitivity of adult male and female *Lepeophtheirus salmonis* populations following on-host exposure to triple dose emamectin benzoate (EMB; SLICE®).

L. salmonis population	Number of sea lice (male and female) [Mean ± SEM]			% Survival		Average % mortality	
						Male	Female
	Day 0	1 dpS	13 dpSC	1 dpS	13 dpSC		
S1-2b	40. 7 (n = 6)	34.6 ± 2.1 (n = 5)	27.6 ± 2.0 (n = 8)	85.1	67.8	53.8	24.4
R2-2a	23.6 (n = 6)	26.6 ± 1.6 (n = 5)	12.2 ± 1.7 (n = 10)	112.7	51.7	36.6	20.8

dpS = days post-commencement of emamectin benzoate treatment; dpSC = days after cessation of EMB treatment.

Table 4.5. Emamectin benzoate (EMB; SLICE®) sensitivity of adult male (EMB resistant) and female (EMB sensitive) *Lepeophtheirus salmonis* crosses following on-host exposure to a triple dose EMB treatment (n = 5).

Days post commencement of SLICE treatment (dpS)	Mean number of sea lice (± SEM)			
	R3-2a (treated)	R3-2a (not treated)	RX2S F1	RX1-3S F1
0	17.0 ± 2.6	26.7 ± 7.7	15.7 ± 2.8	11.0 ± 4.1
7	10.0 ± 1.7	17.2 ± 1.9	1.2 ± 0.5	2.6 ± 0.8

dpS = post-commencement of emamectin benzoate treatment; R3-2a = F3 generation EMB resistant *L. salmonis*; RX2S F1 and RX1-3S F1 = EMB resistant/sensitive crosses of *L. salmonis*.

4.5. Discussion

We investigated locations of sea lice populations across different Atlantic salmon farming regions in the New Brunswick side of the Bay of Fundy and sex differences as factors contributing to EMB resistance in sea lice following off-host (i.e. bioassays and P-gp mRNA analyses) and on-host (i.e. sea lice survival on EMB-treated Atlantic salmon) drug exposure. The EMB bioassay results indicate population, species, sex and temporal differences in EMB EC_{50} values. An earlier study by Westcott et al. (2008) reported EMB EC_{50} values of 37 and 12 ppb for preadult male and female F1 laboratory reared *L. salmonis* respectively, derived from farms (in 2005) prior to confirmed resistance development. Results of Bioassay I (present study) carried out using adult male and preadult female F1 in-house reared *L. salmonis* derived from farms with EMB-resistant sea lice (BMA 1), indicated EC_{50} values of 329 and 304 ppb, respectively. Notwithstanding the differences in EC_{50} values, both studies demonstrated that F1 in-house reared *L. salmonis* were similar in EMB sensitivity as the field F0 generation from which they were derived (Jones et al. 2012) and further confirmed the reduced sensitivity to EMB in recent field and lab-isolated populations of the parasite. Also, EMB EC_{50} values [25 to 118 (CI = 17 - 292) ppb; from 2002 to 2005] reported by Westcott et al. (2008) for preadult sea lice collected from the Bay of Fundy are several fold lower than the current EMB EC_{50} values for BMAs 1 (adult male and preadult female) and 2a (adult male and female) [Table 4.3], evidence for reduced EMB efficacy in some locations within the Bay. It has been observed that differences in sea lice EMB sensitivity can exist within a given region (Westcott et al. 2010, Whyte et al. 2013). Consistent with the BMA 1 EMB EC_{50} values, as well as the EMB EC_{50} values reported in Chapter 2, the 2012 EMB EC_{50} values from Bioassays IIa and IIb (R0a-2a and R1-2a) were significantly higher (> 2-fold) for the adult males compared with adult females

(Table 4.3). Bioassay IIb results also revealed that S0-2b was more sensitive to EMB compared with R1-2a despite one generation difference between both populations of *L. salmonis*. The higher EMB EC_{50} value of BMA 2a sourced *L. salmonis* (R1-2a) is most likely a consequence of drug selective pressure whereby sensitive strains of the parasite are gradually replaced with more resistant strains that were originally in the minority prior to introduction of the sea lice medication. We did not have access to farm treatment records to ascertain whether there were any differences in the number of EMB treatments and/or efficacy between the salmon farms sourced for sea lice in BMAs 2a and 2b. However, Jones et al. (2012) reported that although there was increasing number of sea lice from 2004 to 2008 in the Bay of Fundy, sea lice numbers were lowest in Grand Manan (BMAs 2b and 3b). Jones et al. (2012) also reported that there were fewer EMB treatments and greater efficacy in Grand Manan compared with other salmon farm locations within the Bay. In the follow-up Bioassay IV (present study), the S0-2b progeny (S1-2b) displayed a resistant phenotype. This may have occurred due to: 1) the low EMB sensitivity in *L. salmonis* collected from BMA 2b Atlantic salmon farms not being heritable to the first generation in-house developed parasite or 2) as a result of a relatively lower contribution of EMB susceptible progeny following re-infection and development compared with more EMB resistant strains within the population, consequence of EMB treatment and selection just prior to sea lice collection. Avermectin resistance can develop very quickly compared with other classes of anthelmintics, e.g. F1 progeny from reciprocal crosses between avermectin-sensitive and -resistant parent *Haemonchus contortus* displayed the same level of resistance as the resistant parent (Dobson, Le Jambre & Gill 1996). Also, reciprocal crosses of thiabendazole-resistant and -sensitive parent *H. contortus* yielded F1 and F2 progenies that were nearly as thiabendazole-resistant as their resistant parents (Le Jambre, Southcott & Dash 1976). However, F1 and F2

progenies from reciprocal crosses of cambendazole-resistant and -susceptible strains of *H. contortus* were as sensitive to the parasiticide as the sensitive parent (Herlich, Rew & Colglazier 1981). The foregoing suggests that although Grand Manan sea lice currently respond to EMB treatment relative to the other BMAs (Jones et al. 2012), resistance to the parasiticide may be developing.

On the other hand, the EMB resistant/sensitive *L. salmonis* crosses recorded lower % survival compared with F3 generation EMB resistant *L. salmonis* samples (R3-2a) especially for the female treatment groups (Table 4.3). This is in agreement with the second on-host EMB treatment whereby the crosses had greater sea lice reduction between 0 and 7 dpS compared with the in-bred R3-2a *L. salmonis* samples. Hence, our results suggest that sea lice reared in the laboratory will maintain their EMB sensitivity status for up to three filial generations (Bioassay V). The existence of biological costs to maintaining resistance phenotype in *L. salmonis,* especially for the female parasite, is not known. Also, the number of generations it may take for a resistant population of sea lice to lose its resistance status in the absence of drug exposure is not known. Passage of cambendazole resistant strain of *H. contortus* up to 24 generations did not result in reduced resistance phenotype (Herlich, Rew & Colglazier 1981).

Female *L. salmonis* expressed lower levels of P-gp in both the resistant and sensitive populations (see Fig. 4.3 and discussion further below). How the choice of resistant and sensitive sex (i.e. crossing resistant males with sensitive females or vice versa) for the crossbreeding experiment affects EMB sensitivity of the progeny is not known. We chose to cross sensitive females with resistant males because males have been shown to be the more EMB-resistant sex within both EMB-resistant (Chapter 2, Westcott et al. 2008, Heumann et al. 2012) and -sensitive sea lice populations (present study). Although the pattern of EMB

resistance inheritance in sea lice is not known, avermectin resistance inheritance in the nematode, *H. contortus,* is believed to be completely dominant and under the control of a single gene (Le Jambre et al. 2000). It is unknown whether one or more EMB resistant traits in sea lice are dominant, recessive or sex-linked. Endosulfan resistance was associated with sex-linked alleles in *Helicoverpa armigera* (Daly & Fisk 1998). Inheritance of thiabendazole resistance in *H. contortus* and *Trichostrongylus colubriformis* is semi- and incompletely recessive, respectively (Le Jambre, Royal & Martin 1979, Martin, McKenzie & Stone 1988, Sangster, Redwin & Bjorn 1998). Although maternal influence (sex-link) was reported for thiabendazole resistance inheritance in *T. colubriformis*, the maternal aspects of inheritance of resistance to the parasiticide in *H. contortus* was described as matroclinous. In *T. colubriformis*, when resistance was inherited through the female parent, the trait was incompletely recessive while when through the male parent, it was completely recessive (Martin, McKenzie & Stone 1988). Inheritance of the levamisole resistance trait in *H. contortus* was described as incomplete recessive with no maternal influence or sex linkage (Sangster, Redwin & Bjorn 1998). On the contrary, inheritance of the levamisole resistance trait in *T. colubriformis* is completely recessive and sex-linked (Martin & McKenzie 1990). Studies by Herlich, Rew & Colglazier (1981) suggest that inheritance of cambendazole resistance in *H. contortus* is not sex-linked, and that it could be associated with a heterozygous recessive allele. Understanding how anthelmintic resistance phenotypes are passed from one generation of parasite to the next could be useful in effective drug rotations to control parasite infections (Dobson et al. 1987, Gill & Lacey 1998). The limited range of treatment options and control strategies for several years has contributed to resistance development in sea lice (Denholm et al. 2002). Although the need for chemical diversity and the avoidance of over-dependence on a single chemotherapeutant cannot be over-

emphasized (Sangster 2003, Wolstenholme et al. 2004), the tendency to rely on single products for salmon lice control still exists. This may be traced to the difficulty in developing and licensing new drugs (Denholm et al. 2002) and the insistence of farmers to use "the" drug that works. Emamectin benzoate was initially approved for use in Canadian salmon aquaculture in 1999 by Health Canada Veterinary Drugs Directorate under the Emergency Drug Release program, but did not receive full drug approval until 2009.

At day 0 of the first on-host EMB exposure, the infection level of S1-2b was nearly 2-fold higher than the infection of R2-2a sea lice. This may have caused a relatively higher stress for the respective host (i.e. S1-2b infected group), which may consequently lead to lower EMB exposure of S1-2b compared with R2-2a populations. Although the amount of EMB in the feed was confirmed to be 120 mg L^{-1}, concentration of the drug available to and ingested by the parasite, while feeding on the host, is unknown. Some studies reported increased stress in Atlantic salmon in response to a higher (~100 per fish) sea lice burden (Bowers et al. 2000, Mustafa et al. 2000) and was attributed to development from copepodid to late chalimus and preadult stages. Contrariwise, 10 sea lice per fish for up to 10 days caused increased Na^+/K^+-ATPase activity and plasma chloride ion concentrations compared with 3 and 6 sea lice/fish groups (Nolan, Reilly & Wendelaar Bonga 1999) suggesting that as the number of sea lice on fish increases, and for prolonged periods, level of stress increases which may cause poor feeding and sub-therapeutic EMB concentration in the host.

Caligus elongatus from a suspected EMB-resistant site (BMA 1) displayed >2 fold lower EMB EC_{50} values compared with *L. salmonis* collected previously from the same BMA and from another location known to contain an EMB-resistant sea lice populations (BMA 2a). There are unconfirmed reports that populations of *C. elongatus* at various locations in the Bay of Fundy

fluctuate, but the exact pattern of the fluctuations has not been described. Whether sea lice treatments influence the rise and fall of *C. elongatus* numbers is not known. Outbreaks of *C. elongatus* most likely pre-date those of *L. salmonis* in sea cage culture of salmonids in the Bay of Fundy (Hogans & Trudeau 1989, Hogans 1995). Therefore the fact that *C. elongatus* is relatively EMB-sensitive compared with *L. salmonis* could be linked to the transient presence of *C. elongatus* on salmon in sea cages. This has probably resulted in a reduced exposure history to EMB due to greater opportunity for refuge in wild fish populations for *C. elongatus* compared with *L. salmonis* as has been reported for the latter in Pacific Canada (Jones & Prosperi-Porta 2011, Saksida et al. 2012); consequently causing reduced selection pressure for the development of EMB resistance in *C. elongatus*. *Caligus elongatus* may also lack the ability to develop EMB resistance as rapidly as *L. salmonis*. EMB-resistant *C. rogercresseyi* was reported in Chile several years post-introduction of the parasiticide in Chilean salmon aquaculture (Bravo, Sevatdal & Horsberg 2008) and the emergence of resistant strains of this sea lice species may have been hastened by prior use of ivermectin for controlling the parasite. It is known that drugs of the same class will select for the same resistance mechanism, but whether *P-gp* plays a role in EMB resistance in *Caligus* spp. is yet to be investigated. Given the proposed involvement of *P-gp* in EMB resistant *L. salmonis* (Chapter 2), as well as its role in ivermectin resistance in nematodes (Prichard & Roulet 2007), it is quite possible that the ABC transporter will be involved in EMB resistance in *Caligus* spp.

It is not clear why concomitant exposure of *C. elongatus* to EMB and 10 µM verapamil caused higher EMB EC_{50} compared with EMB exposure alone. The drug resistance reversal agent caused an increase in anthelmintic efficacy against ivermectin and moxidectin-selected strains of *H. contortus* (Molento & Prichard 1999). There was increased mortality in *L. salmonis*

concomitantly exposed to verapamil (at the same concentration used in this study) and EMB compared with EMB exposure alone, previously (Chapter 2). Whether the discrepancy between the effects of verapamil in the present study and earlier report in Chapter 2 is due to species' and/or EMB sensitivity differences is not known. While the study in Chapter 2 employed resistant population of *L. salmonis* (BMA 2a), the present study used a relatively EMB-sensitive population of *C. elongatus*. A previous study demonstrated that benzimidazole resistance reversal effect of verapamil in *H. contortus* is more pronounced in the anthelmintic-resistant strain than in the sensitive strain (Beugnet, Kerboeuf & Gauthey 1997). Verapamil at similar or higher concentrations has been shown to induce P-gp expression in human carcinoma cells [~10 µM verapamil] (Herzog et al. 1993) and LS180 human colonic cell [50 µM verapamil] (Collett, Tanianis-Hughes & Warhurst 2004). The *P-gp* reversal agent may have caused a decrease, instead of increase, in mortality of *C. elongatus* in the present study, potentially due to different sensitivities to the Ca^{++} channel blocker (Nygren & Larsson 1990) and/or through induction of the ABC transporter, consequently decreasing the efficacy of EMB.

Quantitative PCR analyses revealed no significant differences in P-gp mRNA expression between adult female *L. salmonis* samples collected in 2005 and 2012 from BMA 2b, unlike results from other areas unresponsive to the drug (Chapter 2). These findings, combined with the bioassay results for BMA 2b sea lice and treatment efficacy of EMB described by Jones et al. (2012) and Whyte et al. (2013), indicate that Atlantic salmon farms in Grand Manan still contain EMB-sensitive strains of *L. salmonis*. Also, based on the relatively lower EMB EC_{50} reported by Westcott et al. (2008), our findings suggest that resistance is already developing in Grand Manan. However, given that female *L. salmonis* may be a poor indicator of developing EMB resistance within a given sea lice population (Chapter 2), it is difficult to conclusively determine

the relationship with *P-gp* in this area. Previous studies have shown that EMB can induce P-gp mRNA expression in a dose-dependent manner (Chapter 2). Our results suggest the need to consider sex-based differences in the expression of P-gp while monitoring for EMB resistance development in sea lice. The spatial differences between the BMA 2a and 2b (Fig. 4.1) *L. salmonis* populations may account for the differences in relative P-gp mRNA expression between both populations consequently leading to differences in their relative EMB sensitivities as well as in their progenies. The spatial differences may be linked to differences in tidal excursions (Chang et al. 2007) and/or access to refuge (Saksida et al. 2012). The study by Saksida et al. (2012) associated the relatively low EMB EC_{50} values recorded by *L. salmonis* found in British Columbia to influx of sea lice from the wild salmon population refuge which serve to dilute the effects of EMB selective pressure on salmon farm sourced sea lice. Also, although not confirmed, it is possible that the closer proximity of BMA 2a to the mainland compared with BMA 2b, may have caused exposure of BMA 2a sea lice to environmental contaminants (such as industrial wastes and pesticide runoffs from nearby farms) resulting to a higher expression of P-gp mRNA.

Although males are more tolerant to EMB, express P-gp at higher levels, and are more readily induced compared with female *L. salmonis*, the evolutionary link associated with the sex-based differences is unclear. Apart from the reproductive burden imposed on the female towards egg production, the sexual behavior of the parasite is such that the males change sexual partners more readily than the females and are likely to change host more frequently (Stephenson 2012). Hence it may make more biological sense for the male parasite to be more EMB-resistant compared with the female. Although polyandry exists in copepods, females only require a single copulatory event to produce offspring for the remainder of their lives, in combination with

greater movement between hosts by males (Stephenson 2012), probably provide males with greater opportunity to spread their gametic contribution to future generations over a larger spatial scale. This may allow for resistance development to be driven by the males, rather than the female.

The present study has shown that within an area suspected to contain parasiticide-resistant sea lice, there could be micro-populations of the parasite relatively sensitive to the same drug. This is the first assessment of the EMB sensitivity of *C. elongatus* found in the Bay of Fundy. It is interesting and noteworthy, but not unexpected, that EMB-sensitive *C. elongatus* and -resistant *L. salmonis* were located in the same site (BMA 1; Fig. 4.1). More investigations on the nature of the species' differences in EMB sensitivity in sea lice, as well as the heredity of EMB resistance mechanisms, is necessary for a better understanding of how this economically important parasite strives to survive its control.

4.6. References

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1. General discussion

Resistance to sea lice therapeutants has developed rapidly with increased farmed salmon production. As with land-based animal production, prevalence of infection increases with increase in fish density. This makes it easier for a parasite such as sea lice to locate its host, feed and keep multiplying. Drug selection due to prolonged dependence on a specific chemical or drug, in this case emamectin benzoate (EMB), further exacerbates the situation. This is because if a particular drug is continually employed for sea lice control, surviving parasites are favoured to continue propagating, and will eventually become the dominant strain of parasite in the population. Other classes of drugs, such as organophosphates and pyrethroids, which have been used for sea lice control, over time have also become ineffective in some salmon farms (Denholm et al. 2002). This was linked to the emergence of sea lice strains resistant to the respective drugs. The fact that EMB resistance in sea lice, similar to other parasiticides, occurs over time underscores the need to understand the mechanisms of loss of efficacy and investigate sensitive tools to monitor resistance development.

The effectiveness of EMB and its advantages over other sea lice medicines made it the drug of choice with almost exclusive use for sea lice control in salmon farms in the Bay of Fundy, NB (Westcott, Hammell & Burka 2004) and elsewhere. Consequently, the use of EMB has declined considerably due to resistance development.

Numerous studies have linked P-glycoprotein (*P-gp*) with resistance to the closely related ivermectin in nematodes and arthropods (reviewed by Prichard & Roulet 2007). It was hypothesized that over-expression of *P-gp* in the gastrointestinal epithelium would impede the absorption of EMB, ingested by the salmon louse (Tribble, Burka & Kibenge 2007). P-glycoprotein, a member of ATP-binding cassette (ABC) transporters, causes the efflux of diverse

range of molecules from within cells to the exterior (Fojo et al. 1985, Raviv et al. 1990). Although genetic changes in the EMB target GluCl channels have been associated with ML resistance (Njue et al. 2004), increased expression of *P-gp* is thought to be the primary mechanism responsible for loss of sensitivity to MLs in nematodes (reviewed by Prichard & Roulet 2007). Also, *P-gp* appears to play a major role in the protection of aquatic invertebrates from xenobiotics (Smital & Kurelec 1998, Fulton et al. 1999, Lyons-Alcantara et al. 2002). Macrocylic lactones are substrates for *P-gp* and have high binding affinity for the transporter (Lespine et al. 2007). The present study investigated the role of *P-gp* in EMB resistance in sea lice using multiple methods such as P-gp gene expression, competitive inhibition test, and an assay for ATPase activity. The latter two tests indicated that *P-gp* interacts with EMB *in vitro* (Chapter 2). Increased expression of P-gp was linked to ML resistance in *C. elegans* and *H. contortus* (Blackhall et al. 1998a, Xu et al. 1998, Sangster et al. 1999, James & Davey 2009). Using *in situ* hybridization technique, P-gp mRNA was localized in the digestive tract and pharynx of *H. contortus* (Smith & Prichard 2002). Unfortunately, we were unable to detect P-gp at the mRNA and protein levels using *in situ* hybridization, immunohistochemistry, Western blotting and ELISA techniques (Appendix 1). This is likely due to low expression (mRNA and protein) and/or sub-optimal antigenic specificity (protein) of the C219 anti-mouse *P-gp* antibody used. Trypsin was detected in epithelial cells of *L. salmonis* digestive tract following similar tissue handling and processing employed for *P-gp* (Kvamme et al. 2004), and was successfully used as a positive control in the present study.

The *P-gp* competitive inhibition test using MDR-reversing agents, such as verapamil, is a well-established means of determining compounds that are substrates of the efflux transporter. The strategy has been employed for the reversal of *P-gp*-mediated drug resistance in

anthelmintic therapy (reviewed by Lespine et al. 2012). Concurrent exposure of *L. salmonis* to EMB and verapamil caused higher sea lice mortality compared with exposing the parasite to the parasiticide or verapamil alone, indicating that EMB interacts with *P-gp* in the parasite (Chapter 2). This also suggests that verapamil may be interacting at the same site on the efflux pump as EMB. Hence, the increased sensitivity of *L. salmonis* to EMB following concomitant verapamil exposure is most likely a consequence of competitive inhibition of *P-gp* by the MDR-reversal agent, causing an increase in the concentration of EMB in the parasite. However, relatively EMB sensitive *C. elongatus* responded differently to concurrent verapamil/EMB exposure whereby the group that were treated with both drugs recorded higher EMB EC_{50} values compared with the EMB-treatment group. This suggests that the effect of verapamil may be most prominent when used on a resistant population, although potential species differences should be considered as well. Reversal of ivermectin resistance in nematodes using verapamil has been previously reported (Molento & Prichard 2001). Although this is a potential means of enhancing ML efficacy (Bartley et al. 2009), differences in kinetics of different MLs and existing reversal agents and potential toxicity of the latter may hamper realistic use of this strategy in the field (Lespine et al. 2008). Although results indicate that concurrent administration of MDR-reversing agents (such as verapamil) and EMB could increase the sensitivity of sea lice to the parasiticide, more studies are necessary to confirm the possibility of using MDR-reversing agents in the control of sea lice in salmon farms. In the present study, verapamil, a well known calcium channel blocker, caused ≥10% mortality in *L. salmonis* at 10 and 30 µM and 100% mortality at 100 µM, possibly due to blockade of calcium channels in the parasite. Verapamil was used in this study as a pharmacological tool to ascertain the role of *P-gp* in EMB resistance *in vitro*, but would probably not be ideal for field application, as it is not likely to accumulate in

the host skin, limiting availability to the parasite. It may also induce cardiac toxicity to the host salmon due to Ca^{++} channel inhibition. Other ABC-transporter inhibitors with appropriate pharmacokinetics and host and human safety parameters need to be developed.

Resistance is a consequence of changes in the genetic profile of the parasite population and/or selection for resistant strains of parasite that will subsequently lead to a phenotype of reduced sensitivity to treatment (Eng & Prichard 2005). Genes conferring resistance arise through mutation, but maintain very low frequencies in pest populations in the absence of drug exposure (Denholm et al. 2002). Over time and with subsequent treatments, genes conferring resistance are passed from one generation of survivors to another (Sangster 1996, Sangster & Gill 1999). Following each treatment, individuals possessing these genes survive and multiply. Early on, the number of resistant survivors may be low to affect treatment outcome, but over time and, if resistance is unchallenged, the survivors will become the dominant individuals within the population, causing treatment failures (Denholm et al. 2002). More genetically variable organisms possess greater capability of having an allele capable of causing poor treatment outcomes. The evidence for the involvement of a gene in resistance to a drug can be obtained by examining the genetic variability of the gene between individuals sensitive and resistant to the drug (Blackhall et al. 1998b), where available. Variations in resistance to sea lice therapeutants could be due to phenotypic or genetic differences and has been examined using different approaches (Boxaspen 2006). Provided the appropriate genes are investigated, changes in genetic profile are likely to be evident prior to widespread resistance-induced treatment failure and such changes in genetic disposition could be used to monitor the development of resistance to the parasiticide in use (Eng & Prichard 2005). The application of population genetics promises to be a useful tool in the analysis of drug resistance in parasites and other pest species

(Blackhall et al. 1998b). Identifying genes associated with drug resistance selection will be useful in the development of possible markers for tracking reduced drug sensitivity as it develops (Sangster et al. 2002). Monitoring is important for the rotational use of parasiticides so as to delay resistance development (Zhao et al. 2006). Similar to the key factors discussed in the review on monitoring anthelmintic resistance in human onchocerciasis, including host, stage of parasite, and timing of monitoring (Churcher & Basanez 2009), proper monitoring of all stages of sea lice is important for the strategic timing of treatments of farmed salmon (Brooks 2009). Depending on locality/region, reduced sensitivity and potential resistance to currently available medicines are constant threats to the control of sea lice populations on salmon farms. Hence there is the need for ongoing monitoring of treatment efficacy and effects on performance. Monitoring strategies should be precise and practical. They should also be robust, simple and repeatable with an unambiguous endpoint as well as have sufficient sensitivity to detect changes in efficacy of the parasiticide. Bioassays are commonly used to measure clinical effectiveness and have been used to investigate the effects of EMB on gene expression in surviving sea lice (Tribble, Burka & Kibenge 2007). Although the bioassay protocol shows promise as a method to verify clinical resistance, it lacks rapidity and simplicity for use as a routine test (Westcott et al. 2008). Thus, initial work to develop bioassay methods has focused on testing preadult and adult sea lice that have been removed from their hosts. This has several implications. Firstly, once removed, the sea lice can only be used within a limited time frame, usually within 48 h (to avoid stressed samples), for meaningful results. Secondly, endpoints can be hard to define, as the parasites can survive in a moribund stage for prolonged periods following exposure to most control agents (Denholm et al. 2002). Hence the bioassay is best used to establish indicators of sensitivity to the parasiticide under study that is by using concentrations that have been shown to

be effective (Westcott et al. 2008). Since the bioassay must be performed shortly after detaching the parasite from the host, to avoid flawed results due to stressed sea lice, endpoints can be unclear especially between weak and moribund parasites following exposure in this system (Denholm et al. 2002). These shortcomings create the need for alternative methods of monitoring resistance development in the parasite, for example, using molecular tools such as RT-qPCR.

Depending on the prevailing resistance mechanism, developing biochemical assays that measure quantitative/qualitative changes in enzymes conferring resistance, or molecular techniques (Elard, Cabaret & Humbert 1999, von Samson-Himmelstjerna & Blackhall 2005) that detect genetic changes in drug resistant parasites, is a sensible direction to follow as part of the overall parasite control strategy (Denholm et al. 2002). Genetic changes in the structure or expression of P-gp (Sangster et al. 1999) may provide useful markers for monitoring resistance in parasites under prolonged avermectin treatment (Eng & Prichard 2005). Macrocyclic lactones can induce over-expression of P-gp in parasites and changes in the expression of this ABC transporter could be monitored as a means of detecting EMB resistance in *L. salmonis* on salmon farms. In the present study, EMB induced over-expression of *L. salmonis* P-gp mRNA in a concentration-dependent manner (Chapters 2 and 4). The upward trend in the relative P-gp mRNA expression in archived *L. salmonis* and the fact that P-gp mRNA expression for the March 2011 samples was significantly (> 2-fold) higher than the expression for 2002, 2008 and 2010 samples suggest that P-gp mRNA expression level was increasing over the years. Unfortunately, only adult female parasites were archived and analyzed; otherwise, it would have been interesting to investigate the changes in the expression of the transporter in the male parasite over the same period. This is because *L. salmonis* males are less sensitive to EMB and

have a higher level of P-gp expression, hence would likely be a better model for tracking development of EMB resistance compared with the female parasites (Chapter 2). All the same, results indicate that RT-qPCR could be employed to monitor EMB resistance development in *L. salmonis* and should be an integral and routine part of resistance detection and management, similar to what has been done with parasitic nematodes (Humbert et al. 2001, Sangster 2001, von Samson-Himmelstjerna 2006).

A putative *L. salmonis* P-gp was initially cloned (Tribble et al. 2008); however, with the cloning and addition of more P-gp sequences to the GenBank database, the putative ABC transporter reported by Tribble et al. (2008) was discovered to be ABCB8 (Heumann et al. 2012). ABCB8 is a mitochondrial half-transporter speculated to be involved in intracellular transport of iron and sulphur (Burke & Ardehali 2007). ABCB8 has been shown to be involved in melanoma resistance to doxorubicin (Elliott & Al-Hajj 2009). As a follow-up on initial reports of EMB induced concentration-dependent differential expression of ABCB8 in *L. salmonis* (Tribble, Burka & Kibenge 2007), the effect of EMB on cellular respiration of *L. salmonis* using isolated mitochondria was investigated (data not shown). Unfortunately, transitioning from state 3 to state 4 respiration was not achieved; hence we were unable to determine possible effects of the parasiticide on cellular respiration in the parasite.

Widespread parasiticide resistance in *L. salmonis* underscores the need for an integrated sea lice management strategy achievable through combined use of drugs, chemicals, and non-chemical alternatives including manipulation of host immunity using vaccines and/or immunostimulants (Raynard et al. 2002). Innate immunity provides protection against pathogen invasion in fish (Magnadóttir 2006, Whyte 2007) and its manipulation is currently being explored as a sea lice control strategy. Inflammation, cellular infiltration and hyperplasia are the

3 main hallmarks of effective innate responses to sea lice infection. Chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon mount inflammatory and hyperplastic responses at the sites of sea lice attachment and are able to reject the parasite shortly after infection (Johnson & Albright 1992, Braden et al. 2012). However, Atlantic salmon lack such effective responses (Fast et al. 2003) despite induction of innate immune response genes (Skugor et al. 2008, Tadiso et al. 2011), generating interest in inducing innate immune responses of Atlantic salmon as a possible control strategy against sea lice infection. Boosting host immunity against invading pathogens and parasites through immunostimulation has been employed for management of numerous infections in several commercially produced aquatic species, including the salmonids (Ringo et al. 2011). However, the effects of prior host immunostimulation on chemotherapy are not well understood and may not always offer protection against a pathogen (Kunttu et al. 2009). In the present study, sea lice with different immunostimulant backgrounds were subjected to a 24 h EMB bioassay. Contrary to our prediction, the parasites associated with immunostimulant-fed salmon did not significantly differ with control values within each stage and sex category and had higher levels of P-gp. Some studies have shown that acute and chronic inflammatory responses can increase or decrease P-gp expression, respectively (Dumoulin et al. 1997, Ho & Piquette-Miller 2006). Although inducing inflammation promotes the rejection of sea lice, especially in Pacific salmonids (Johnson & Albright 1992, Jones, Kim & Bennett 2008, Wagner 2008), concurrent administration of EMB and immunostimulants to Atlantic salmon may, in fact, favour resistance development to the drug, which may thus limit the use of immunostimulants in combination with certain therapeutants. This also indicates species differences in response to therapy among the salmonids.

Within a given *L. salmonis* population, males were observed to be more EMB resistant compared with females (Westcott et al. 2008, Heumann et al. 2012), but this sex-based difference in the parasite's EMB sensitivity is not well described. Such knowledge could be exploited in tracking EMB resistance development in salmon farms more efficiently. For example, findings from the present study (Chapter 3) suggest that laboratory-reared *L. salmonis* will maintain EMB resistance phenotype for up to 3 filial generations and that inheritance of resistance to the parasiticide may be through the male parasite.

Although there are reports of sea lice tolerance to EMB on fish farms in the Bay of Fundy (Westcott et al. 2008), some sea lice populations within this region are still relatively EMB-sensitive compared with sea lice populations at other salmon farm locations within the region (Jones et al. 2012). We investigated locations of sea lice populations across different salmon farming areas in the Bay of Fundy and sex-based differences as factors contributing to EMB resistance in sea lice (Chapter 4). Results indicate population, species, sex-based and temporal differences in EMB EC_{50} values confirming presence of micro-populations of the parasite relatively sensitive to the drug. Also, EMB-sensitive *C. elongatus* and -resistant *L. salmonis* were located in the same site, confirming species differences in EMB sensitivity and could indicate existing differences in refuge sea lice populations between both parasite species. In contrast, there is a lack of resistance of sea lice to EMB on Pacific Canadian salmon farms, likely due to the presence of *L. salmonis* originating from a large wild salmon population in Pacific Canada, diluting any rise in EMB-resistant sea lice populations (Saksida et al. 2012). Also, threespine sticklebacks (*Gasterosteus aculeatus*) have been reported to be a refuge for sea lice in Pacific Canada (Jones & Prosperi-Porta 2011). The wild salmon population in the Atlantic has declined considerably (Anderson, Whoriskey & Goode 2000) and threespine sticklebacks are not

known to be infected with *L. salmonis* in this region, decreasing the possibility of EMB-sensitive non-farm sea lice from diluting any rise in EMB-resistant sea lice population in the Atlantic. It is not known whether sea lice have any other refuge in Atlantic Canada.

5.2. Conclusion and future directions

It is evident that drug rotations together with non-chemical alternatives are necessary for effective sea lice management. This will entail a sensitive means of early detection of drug resistance and making necessary changes to the treatment used, thereby disrupting the rise of parasiticide-resistant sea lice populations. Drug resistance management in sea lice control requires effective implementation of multi-faceted integrated pest management strategies (Sangster 2001). However, given our findings on possible effects of prior host immunostimulation on EMB efficacy, possibly via upregulation of P-gp, caution has to be exercised in the implementation of integrated sea lice management strategies. The salmon aquaculture industry should avoid over-reliance on single therapeutants. Regulatory bodies should be prudent in approving new compounds such that there are several alternative sea lice therapeutants with different modes of action available at any given in time. Also, drug companies should be keen on developing products with different mechanisms of action (Denholm et al. 2002).

Finally, although some studies have shown that MLs interact with other ABC transporters, *P-gp* is believed to be the major resistance mechanism for this family of parasiticides (Prichard & Roulet 2007, Kerboeuf & Guégnard 2011). Results of the present study, especially the *P-gp* competitive inhibition test results, strongly indicate that the efflux transporter is involved in reduced sensitivity of *L. salmonis* to EMB. However, there is a need to characterize P-gp mRNA and protein *in situ* using more sensitive tools specifically developed for

L. salmonis. Changes in the expression of resistance-associated genes, such as those for *P-gp*, can be monitored and used in the diagnosis of resistance development to parasiticides. The present study has demonstrated that RT-qPCR can be employed in monitoring resistance development to EMB. Knowledge of the timing for resistance development will inform necessary changes to treatment options to prevent treatment failure. Further comparisons between EMB-sensitive and -resistant strains of *L. salmonis* may be necessary to verify the degree to which *P-gp* is responsible for the loss of parasite sensitivity to the drug. More investigations on the nature of the species differences in EMB sensitivity in sea lice, as well as the heredity of EMB resistance mechanisms, is necessary for a better understanding of how this parasite strives to survive its control.

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APPENDIX 1

THE SEARCH FOR P-GLYCOPROTEIN EXPRESSION IN *LEPEOPHTHEIRUS SALMONIS*

6.1. Summary

This section describes work done to detect *L. salmonis* at the mRNA and protein levels and to quantify the protein expression. This is important because knowledge of where P-gp is expressed in the parasite could assist in understanding the role of the transporter in sea lice resistance to EMB. The ability to determine *P-gp* levels in different sea lice populations and sexes will be helpful in tracking EMB resistance development in the parasite.

6.2. Introduction

Resistance development to the macrocyclic lactone (ML), emamectin benzoate (EMB; the major sea lice medicine for about a decade), has been a major concern for *L. salmonis* control due to over-reliance on the parasiticide by salmon farmers (Westcott, Hammell & Burka 2004). Consequently resistance development to the drug by *L. salmonis* has been reported in Atlantic Canada and Europe (Hjelmervik et al. 2010, Westcott et al. 2010) and in *Caligus rogercresseyi* in Chile (Bravo, Sevatdal & Horsberg 2008, Horsberg 2012). P-glycoprotein, a member of ABC transporters that belong to integral plasma membrane proteins, cause the efflux of a diverse range of molecules from within cells to the exterior (Fojo et al. 1985, Raviv et al. 1990). Although genetic changes in GluCl channels have been associated with ML resistance (Njue et al. 2004), increased expression of P-gp is widely believed to be the primary mechanism responsible for loss of parasite sensitivity to MLs (reviewed by Prichard & Roulet, 2007). P-glycoprotein plays a major role in the protection of aquatic invertebrates from xenobiotics (Smital & Kurelec 1998, Fulton et al. 1999, Lyons-Alcantara et al. 2002). Macrocylic lactones have been shown to be substrates for *P-gp* (Lespine et al. 2007) and increased expression of P-gp

was linked to ML resistance in *Caenorhabditis elegans* and *Haemonchus contortus* (Blackhall et al. 1998, Xu et al. 1998, Sangster et al. 1999, James & Davey 2009).

Several studies have associated *P-gp* with resistance to the closely related ivermectin in invertebrates. P-glycoprotein mRNA was localized in the digestive tract and pharynx of *H. contortus* (Smith & Prichard 2002). It is hypothesized that up-regulation or over-expression of P-gp in epithelial cells of *L. salmonis* digestive tract would serve to limit the absorption of EMB, ingested by the salmon louse (Tribble, Burka & Kibenge 2007). This study investigates the abundance and expression pattern of P-gp mRNA and protein in *L. salmonis* using *in situ* hybridization, immunolocalization, ELISA and Western blot techniques. We hypothesize that EMB resistance in *L. salmonis* is due to increased *P-gp* expression in epithelial cells of the parasite digestive tract. Experiments to localize P-gp in epithelial cells of the parasite digestive tract at the protein and mRNA levels were carried out using immunohistochemistry and *in situ* hybridization techniques, respectively. Differential levels of *P-gp* in archived sea lice populations were investigated using ELISA and Western blot techniques to determine temporal increase in the expression of the transporter.

6.3. Materials and methods

6.3.1 Chemicals

The chemicals used for this study were of analytical grade and purchased from Sigma-Aldrich, St. Louis, MO, unless stated otherwise.

6.3.2. Immunolocalization

Immunolocalization of *P-gp* in *L. salmonis* was conducted according to the method described in Tribble et al. (2008). Briefly, adult male and female *L. salmonis* collected from

Atlantic salmon farms in the Bay of Fundy in July 2009 were transported back alive to the laboratory in cold (10°C) seawater, and were fixed in 10% buffered neutral formalin for 24 h at room temperature (RT). The samples were then dehydrated with increasing concentrations of ethanol (70%, 95% and 100%), cleared in xylene, and then embedded in paraffin. The embedded samples were cut into 5 µm-thick sections, mounted on positively charged slides, allowed to air-dry at RT, and then stored at RT until further use.

Prior to rehydration for immunolocalization, the sections were deparaffinized in xylene and then incubated in 0.3% hydrogen peroxide in 100% methanol with shaking. The sections were then rehydrated using decreasing ethanol concentrations (100% for 30 min, and 95%, 70%, 50% and distilled water for 2 min each). The samples were rinsed with phosphate-buffered saline (PBS) and incubated with 10% normal horse serum (Vector Laboratories, Burlingame, CA) in PBS for 20 min to block for non-specific binding of the secondary antibody. Primary mouse monoclonal (C219) antibody (Abcam, Cambridge, MA) specific for mammalian *P-gp* was then applied to the slides empirically at 1:20 and 1:40 dilutions in PBS, and incubated for 12 h at 4°C. Following the overnight incubation, the slides were first rinsed and then washed with PBS for 10 min with shaking. Secondary antibody was applied to the slides and incubated for 1 h at RT. The PBS rinse and wash were repeated and a solution made up of DAB, $NiCl_2$ and H_2O_2 applied on the slides. The slides were rinsed and washed with tap water and then dehydrated in increasing ethanol concentrations (50%, 70%, 95% and 100%) and xylene. Following the dehydration, the sections were covered with a cover slide and viewed for immunoreactions.

6.3.3. SDS-PAGE with Western blotting

Archived adult female sea lice samples (collected in 2002, 2004, 2005, 2008 and 2010 and stored at -80°C) were tested for differential *P-gp* expression. Briefly, 3 or 4 adult female *L. salmonis* were randomly selected from each group (year of collection) and placed in a plastic 5 mL tube containing 1.5 mL ice-cold 0.1 M PBS and homogenized. The homogenate was centrifuged at 300 x *g* for 20 min at 4°C. The supernatant were collected and centrifuged at 20,000 x *g* at 4°C for 30 min. The resultant pellet was dissolved in PBS and the protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON). Fifty to 100 µg of protein were separated on a 8% SDS-polyacrylamide gel at 100 V for ~45 min. The separated protein was then transferred to nitrocellulose membrane at 100 V for 1 h. Afterwards, the nitrocellulose was placed face-up in a plastic container and the protein blocked with excess 5% skim milk in 0.2% Tris-PBS (T-PBS) for 1 h at RT with rocking. This was followed by PBS wash. C219 and JSB1 primary antibodies [(Abcam) specific for mammalian *P-gp*], reconstituted (1:500) in T-PBS (separately) were applied to the nitrocellulose membrane and incubated for 1 h at RT with rocking. The nitrocellulose was washed again with 0.1 M PBS. Secondary antibody conjugated with Horseradish peroxidase (HRP), reconstituted in T-PBS, was applied to the nitrocellulose and then incubated for 1 h at RT with slow agitation. Excess secondary antibody-HRP was rinsed off the nitrocellulose membrane using 0.1 M PBS. Chemiluminescent detection of HRP was performed using an ECL Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol. MDR1 MDCK II (Madin-Darby canine kidney) cell line (over-expressing *P-gp*), salmon liver and upper intestine, and lobster mid-intestine served as positive controls. The MDCK II cell lines (MDR1 and parental) were kindly donated by Dr. Alfred Schinkel (Division of Molecular

Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands). All the tissue samples were collected fresh from the donor animals, were immediately frozen and then stored at -80°C prior to further use. Sample preparations for Western blotting were done at ≤0°C to minimize tissue degradation.

6.3.4. ELISA

A total of 12 snap-frozen adult female *L. salmonis* (collected from Atlantic salmon farms in the Bay of Fundy in February 2010) weighing ~0.3 g were placed in 2 mL of ice-cold 0.1 M PBS and homogenized. In separate tubes, pooled sea lice gut and whole sea lice without the gut were homogenized in 2 mL of ice-cold 0.1 M PBS. Freshly collected mouse liver was processed similarly and served as a positive control. All the tissues were processed on ice. The homogenate was centrifuged at 300 x *g* for 20 min. The resultant supernatant was collected and centrifuged at 20, 000 x *g* for 30 min. Both centrifugation steps were carried out at 4°C. The pellet formed was re-suspended in PBS. Protein concentration was determined for each sample using the Bio-Rad protein assay. The samples were made up to four different 100 µL volumes by the addition of 0, 25, 50 and 75 µL 0.1 M PBS. Detection of *P-gp* was carried out using a *P-gp* ELISA kit (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions. Briefly, a 2-fold *P-gp* concentration standard ranging from 0.156 to 10.0 ng mL^{-1} was prepared using the supplied stock solution. One hundred microlitres of the standard, standard diluent and samples were added to respective wells pre-coated with biotin-conjugated polyclonal antibody specific to mouse *P-gp*, in duplicate. The wells containing standard diluent served as the blank. Plate sealer was applied and the plate was then incubated for 2 h at 37°C. After this initial incubation, the liquid in each well was carefully removed and 100 µL of the supplied detection reagent A containing biotin-conjugated polyclonal antibody specific to mouse

P-gp was added to each well. The plate was sealed and incubated at 37°C for 1 h. After removing the detection reagent A, each well was washed 3 x with 400 µL of the supplied wash solution. The wells were drained of the wash fluid by inverting the plate on absorbent paper. Hundred microlitre of supplied detection reagent B containing Avidin- conjugated HRP was then added to each well. The plate was then sealed and incubated for 30 min at 37°C. Reagent B was then removed from each well and the wells washed with the wash solution supplied. Ninety microlitre of the substrate solution was added to each well. The plate was then sealed and incubated for 25 min in the dark. The enzyme-substrate reaction was terminated by the addition of 50 µL of stop solution containing 3,3',5,5'-Tetramethylbenzidine (TMB) to each well and the plate was immediately read (to determine colorimetric change) at 450 nm on a BioTek® ultra microplate reader (BioTek Instruments, Winooski, VT). Only the wells containing *P-gp* and biotin-conjugated polyclonal antibody specific to *P-gp* will show a change in color. Concentration of *P-gp* in each sample was determined by comparing optical density of the samples to the standard curve.

6.3.5. *In situ* hybridization

6.3.5.1. Production of RNA probe

6.3.5.1.1. Generation of PCR product

RNA was extracted (separately) from adult male and female *L. salmonis* (collected live from Atlantic salmon farms in the Bay of Fundy in May 2011) using the RNeasy® Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and then reverse transcribed following standard procedure. Briefly, one (female) or two (male) *L. salmonis* were placed in a 5 mL plastic tube containing 1 mL Trizol and homogenized using a hand-held

electrical tissue homogenizer. The quality of isolated RNA samples was verified with Experion™ RNA StdSens Chips (Bio-Rad Laboratories). The RNA concentration and the 260/280 nm ratio were confirmed using the Nanodrop 1000 Spectrophotometer (NanoDrop Products, Wilmington, DE). The samples were then stored at -80°C prior to further use. Subsequently, 1 µg RNA of each sample was treated with DNase I (Invitrogen, Carlsbad, CA) and then reverse transcribed using SuperScript® III (Invitrogen) to generate cDNA. PCR reactions were then carried out using *Taq* 2 x Master Mix (New England BioLabs Inc., Ipswich, MA). Briefly, 1 µg DNA template was added to 25 µL of the Master Mix; 1 µL each of forward and reverse primers [designed based on the SL-PGY1 sequence (Heumann et al. 2012) using the Primer 3 software on the NCBI website: **http://www.ncbi.nlm.nih.gov/tools/primer-blast/**], and the reaction volume was brought up to 50 µL total volume. The PCR was done at the following cycling conditions: initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 30 s, 55°C for 30 s and 70°C for 30 s, with a final extension at 72°C for 10 min. The reaction was then terminated at 4°C. The size of the PCR product was confirmed using gel electrophoresis.

6.3.5.1.2. Cloning of PCR product

The PCR product was cloned into a PCR®4-TOPO® vector (Invitrogen) following manufacturer's instruction as follows. To 2 µL of the PCR product, 1 µL salt solution, 2 µL molecular grade water and 1 µL of the vector were added. The mixture was shaken briefly and incubated at 22°C for 5 min and placed on ice prior to transformation.

6.3.5.1.3. Transformation of plasmids into TOP10 *Escherichia coli* cells

Escherichia coli cells were incubated with 2 µL cloning reaction for 20 min. Then the reaction was heat-shocked at 42°C for 30 s and transferred to ice. Two hundred and fifty

microlitre of supplied S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the reaction. The tube containing the reaction was then capped, and incubated with horizontal shaking (200 rpm) at 37°C for 1 h. Thereafter, the mixture was spread on pre-warmed (37°C) lysogeny broth (LB) agar plate containing 100 µg mL^{-1} Ampicillin and 50 µg mL^{-1} Kanamycin. While the control LB agar plate was spread with 50 µL S.O.C. medium only, 2 agar plates were spread with 30 µL diluted (10 µL cells + 20 µL S.O.C.) and 50 µL undiluted mixture of the transformed cells. The three plates were then incubated overnight (~12 h) at 37°C.

6.3.5.1.4. Selection and analysis of colonies by PCR

Distinct colonies were carefully marked, picked, and added to the PCR master mix, as described in sub-section 5.3.5.1.1., and the PCR reactions carried out under the same conditions described therein. Positive transformants were confirmed using gel electrophoeresis and 5 distinct colonies were inoculated into separate tubes containing LB medium. The tubes were incubated overnight (~12 h) at 37°C with shaking.

6.3.5.1.5. Isolation of plasmid DNA

All the steps for isolation of the plasma DNA were carried out at RT. Following the overnight incubation, 1.5 mL of cell suspension was collected from the LB broth and centrifuged at 12,000 x *g* for 3 min to form a pellet. The remaining LB broths were stored in 40% fresh glycerol at -80°C for future use. The entire medium was removed from the isolated pellet and the isolated DNA was purified using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. Briefly, the isolated pellet was resuspended using 250 µL of the supplied Resuspension Buffer (R3) with RNase A, ensuring that no clumps were

formed. Two hundred and fifty microlitre of the supplied Lysis Buffer (L7) was added to the mixture; the tube was capped and gently mixed by inverting it 5 x. The tube was then incubated for 5 min at RT, after which 350 µL of supplied Precipitation Buffer (N4) was added and the contents mixed by inverting or shaking (for large pellets) to form a homogenous suspension. The suspension was then centrifuged at 12,000 x *g* for 10 min to remove lysis fragments from the lysate. The isolated supernatant was then put into the supplied Spin Column and spun at 12,000 x *g* for 1 min and the resultant solution was discarded. Seven hundred microlitre of supplied Wash Buffer (W9; ~70% ethanol) was added to the tube and centrifuged at 12,000 x *g* for 1 min. Again, the supernatant was discarded; residual Wash Buffer was eliminated from the column by repeating the last centrifugation and the resultant supernatant discarded. DNA within the column was eluted by adding 75 µL preheated (70°C) supplied TE Buffer to the column, incubating for 1 min and centrifuging at 12,000 x *g* for 2 min. The concentration and the 260/280 nm ratio of DNA in the resultant solution were done using Nanodrop 1000 Spectrophotometer (NanoDrop Products). Fifteen microlitre of the mixture containing DNA was sequenced to confirm the orientation of the DNA insert within the plasmid DNA. The remaining DNA samples were stored at -80°C for DIG (digoxigenin) labeling.

6.3.5.1.6. Linearization of plasmid DNA

Positive clones selected from the sequencing result were linearized using NOTI and Pst1 enzymes (Thermo Fisher Scientific, Inc. Waltham, MA) targeting the T3 and T7 promoters on the plasmid DNA, yielding DNA templates for making the antisense and sense RNA probes, respectively. Briefly, 10 µg DNA sample was combined with 10 µL of the enzyme (T3 and T7, separately), gently mixed and briefly spun to bring contents to the bottom of the tube. Samples were then incubated at 37°C for 35 min (NOT1) or 10 min (Pst1). NOT1 digestion was

terminated by heating the tube at 80°C for 5 min prior to purification of the product while for the Pst1 reaction heat inactivation of the enzyme was omitted.

6.3.5.1.7. Purification of linearized plasmid DNA

Following linearization of the plasma DNA, the DNA samples were column-purified using the QIAquick® PCR Purification Kit (Qiagen Sciences, Germantown, MD) according to the manufacturer's instructions. All the centrifugation steps were conducted at 17,900 x *g* for 1 min at RT. Briefly, 5 volumes of the supplied Buffer PB were added to 1 volume of the PCR reaction and then mixed; yellow colour indicated optimal pH (≤7.5) of the solution. The mixture was transferred to the supplied column and centrifuged. Thereafter, 750 µL of supplied Buffer PE (~71% ethanol) was added to the column and centrifuged. The resultant solution was discarded and excess buffer was eliminated from the column by repeating the last centrifugation. The column was placed in a clean 1.5 mL tube, and 30 µL of supplied Elution Buffer was added to the column, incubated for 1 min at RT, and then centrifuged. The concentration and 260/280 nm ratio of DNA in the resultant solution were done using Nanodrop 1000 Spectrophotometer (NanoDrop Products).

6.3.5.1.8. DIG labeling

RNA probe specific to *L. salmonis* P-gp mRNA was generated using the DIG RNA Labeling Kit (Roche Diagnostics, Laval, QC) according to the manufacturer's instructions. To 1 µg of purified DNA template, 2 µL 10x NTP labeling mix, 2 µL 10x Transcription Buffer, 1 µL Protector RNase inhibitor and RNA Polymerase (T3 and T7, separately) were added and the reaction was brought up to 20 µL final volume with water. The solution was gently mixed and incubated for 2 h at 37°C. Two microlitre of supplied DNase I was then added to the reaction

and incubated for 15 min at 37°C to eliminate any contaminating DNA. The DNase treatment was terminated by addition of 2 µL 0.2 M EDTA (pH 8.0) to the reaction. One microlitre of the reaction was removed for RNA gel analysis. Two microlitre of 8 M LiCl and 75 µL 95% ethanol were added to the remaining reaction, mixed and stored overnight (~12 h) at -80°C.

6.3.5.1.9. Hydrolysis of RNA probe

To reduce the RNA probe from 561 bp to an optimal length of 250 bp, hydrolysis reaction was performed based on the following equation:

$$t = \frac{L_0 - L_f}{K \times L_0 \times L_f}$$

t = incubation time in minutes

L_0 = initial length of transcript (in kb)

L_f = desired probe length (in kb)

K = constant (0.11 kb/min at 60°C)

$$t = \frac{0.561 - 0.250}{0.11 \times 0.561 \times 0.250}$$

$$= \frac{0.311}{0.0154275} = 20.16 \text{ min}$$

The RNA probe was removed from the overnight -80°C storage and centrifuged at 13,000 x *g* for 30 min at 4°C. The supernatant was decanted off and the probe was washed with 70% ethanol using centrifugation at 13,000 x *g* for 10 min at 4°C. Ethanol supernatant was poured off and RNA probe was air-dried. One hundred and eighty microlitre of DEPC treated-water was added to the tube and kept on ice. Fifteen minutes later, 20 µL of hydrolysis buffer (40 mM $NaHCO_2$, 60 mM Na_2HCO_3) was added to the tube, incubated at 60°C for ~20 min and

then placed on ice. Hydrolysis neutralization buffer was then added to the RNA probes, mixed and stored at -80°C for at least 30 min. Following the cold storage, the tube was centrifuged at 13,000 x *g* for 30 min at 4°C and then washed with 100 µL 70% ethanol at 13,000 x *g* for 10 min at 4°C. The ethanol was discarded, resultant pellet was air-dried for 15 min and resuspended in 100 µL hybridization buffer [50% formamide, 1 x Denhardt's solution, 5 x SSC (saline-sodium citrate), 100 µg mL^{-1} Heparin, 0.1% Tween 20, 0.1% CHAPS (3-{(3-cholamidopropyl) dimethylammonio}-1-propanesulfonate), 1 mg mL^{-1} Torula RNA)] and stored at -80°C until further use.

6.3.5.2. Fixation and processing of *L. salmonis* tissue

Adult male and female *L. salmonis* collected from Atlantic salmon farms in the Bay of Fundy in May 2011, were fixed in 10% neutral buffered formalin (4% formaldehyde) in 1x fish saline (175 mM NaCl, 2.7 mM KCl, 0.64 mM $MgCl_2.6H_2O$, 2.74 mM $CaCl_2.2H_2O$, 2.22 mM D-glucose 6-phosphate, 3.0 mM Tris HCl) (Valerio, Kao & Fletcher 1992) for ~18 h at 4°C with gentle rocking. Sea lice samples were then dehydrated with increasing concentration of ethanol (70%, 95% and 100%), xylene and then embedded in paraffin. The embedded samples were cut into 7 μm sections, mounted on positively charged slides, baked at 60°C for 1 h and then stored at RT in the dark until further use.

From this point on, all equipment, including glassware and jars used for tissue handling, was decontaminated for RNase either by autoclaving (>230°C) or cleaning with ^{D}R NAse FREE^{TM} (Argos Technologies, Elgin, IL).

6.3.5.3. RNA probe hybridization

The tissue sections chosen for probe hybridization were deparaffinized in xylene twice, 10 min each at RT. The sections were then rehydrated through decreasing concentration of ethanol (100%, 95%, 70%, and 50%) and 1x fish saline, 2 min each at RT. Predigestion of the tissue sections was conducted using 2.5 µg mL^{-1} Proteinase K (Roche Diagnostics) in 1x fish saline for 30 min at 37°C to render the samples permeable to RNA probe. The samples were then incubated in 2 mg $mL^{-}1$ glycine in 1x fish saline for 2 min at RT to terminate the action of Proteinase K. Treatment with 0.1 M triethanolamine pH 7.5 was then done for 5 min at RT. This was repeated (with addition of 0.25% acetic anhydride to the buffer) for 10 min at RT. The sections were then rinsed in 2x SSC and dehydrated using increasing ethanol concentration (50%, 95%, and 100%) for 2 min each at RT. The tissue sections were air dried for 2 h at RT. 50 µL of diluted RNA probe [(5 µL RNA probe and 45 µL hybridization buffer) sense and antisense] were added on the sections, cover slips were placed over the sections, and then incubated in a humid chamber (soaked with 50% formamide in 2x SSC) overnight (~12 h) at 45°C in a water bath.

6.3.5.4. Detection of hybridization reaction

To dislodge the cover slips, tissue sections were immersed in 2x SSC for 5 min at RT followed by incubation in 50% formamide in 2x SSC for 1 h at 45°C. The slides were rinsed in 10 mM Tris-HCl (pH 8) with 500 mM NaCl, made up to 50 mL total volume using molecular grade water for 2 min at RT. This was repeated but with the addition of 0.002 µg $µL^{-1}$ RNase A and 0.01 U $µL^{-1}$ RNase T_1 for 30 min at 37°C. The slides were washed in decreasing salt concentration- 2x, 0.5x and 0.1x SSC, each for 30 min at 45°C. Following the stringent washes, the slides were rinsed in 0.1 M Tris-HCl (pH 7.5) with 150 mM NaCl for 2 min at RT. This was

repeated with the addition of 1% BSA (bovine serum albumin) and 10% lamb serum for 30 min at RT to block non-specific binding sites in the tissue. To detect DIG, 500 µL sheep anti-DIG-alkaline-phosphatase (1:100 of blocking buffer) was applied on each slide and incubated for 30 min at RT. The slides were washed with blocking buffer containing 0.1% Tween 20 and 1% BSA for 30 min, with gentle rocking, and then rinsed in chromogenic buffer [4 mL 1M Tris-HCl (pH 9.5), 1 mL 1M $MgCl_2$, 0.8 mL 5M NaCl, 40 µL Tween 20, 9.68 mg levamisol; brought to 40 mL total volume] for 15 min at RT. Detection of the antibody was done by incubating the slides in chromogenic buffer containing 1.6% Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate [(NBT/BCIP); Roche] for 30 min at RT. NBT/BCIP reaction was eliminated by immersing the slides in tap water for 2 min at RT and the sections were counterstained with Nuclear Fast Red (Vector Laboratories) for 3 min at RT. Following a 2 min wash in tap water at RT, the tissue sections were rapidly dehydrated using increasing concentrations of ethanol (50%, 95%, and 100%) and twice in xylene, all at RT. Cover slip was placed on each slide and then viewed under the microscope.

6.4. Results

6.4.1. Immunolocalization

Immunoreactions were not observed for all the tissues examined except salmon liver bile canaliculi (Figs 5.1-5.2).

6.4.2. SDS-PAGE with Western blotting

P-glycoprotein was detected by Western blot in salmon liver and MDR1 MDCK II cell lines but not in sea lice (Fig. 5.3).

6.4.3. ELISA

Concentration of *P-gp* detected from whole sea lice, pooled sea lice gut, sea lice without the gut, and mouse liver were 0.32, 0.06, 0.13 and 3.43 ng mL^{-1} respectively.

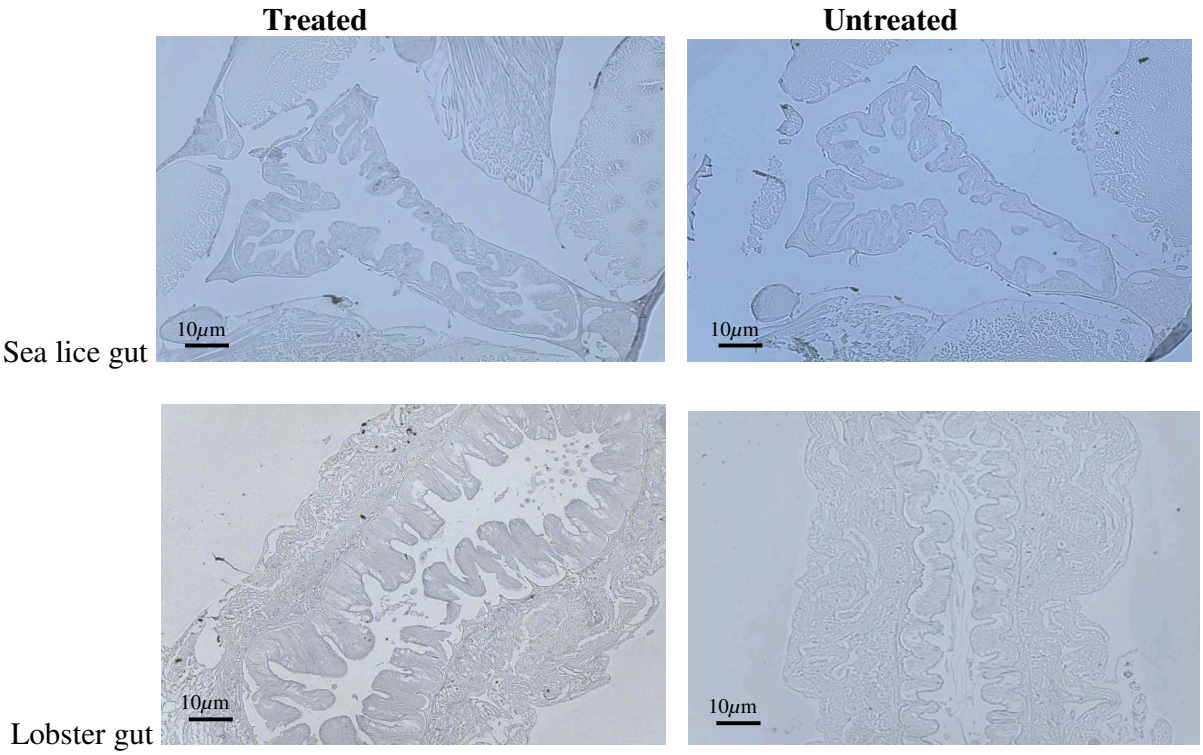


Figure 6.1. P-glycoprotein antibody (C219)-treated and -untreated *Lepeophtheirus salmonis* and *Homarus americanus* mid-gut tissues. Positive immunoreactions were not detected.

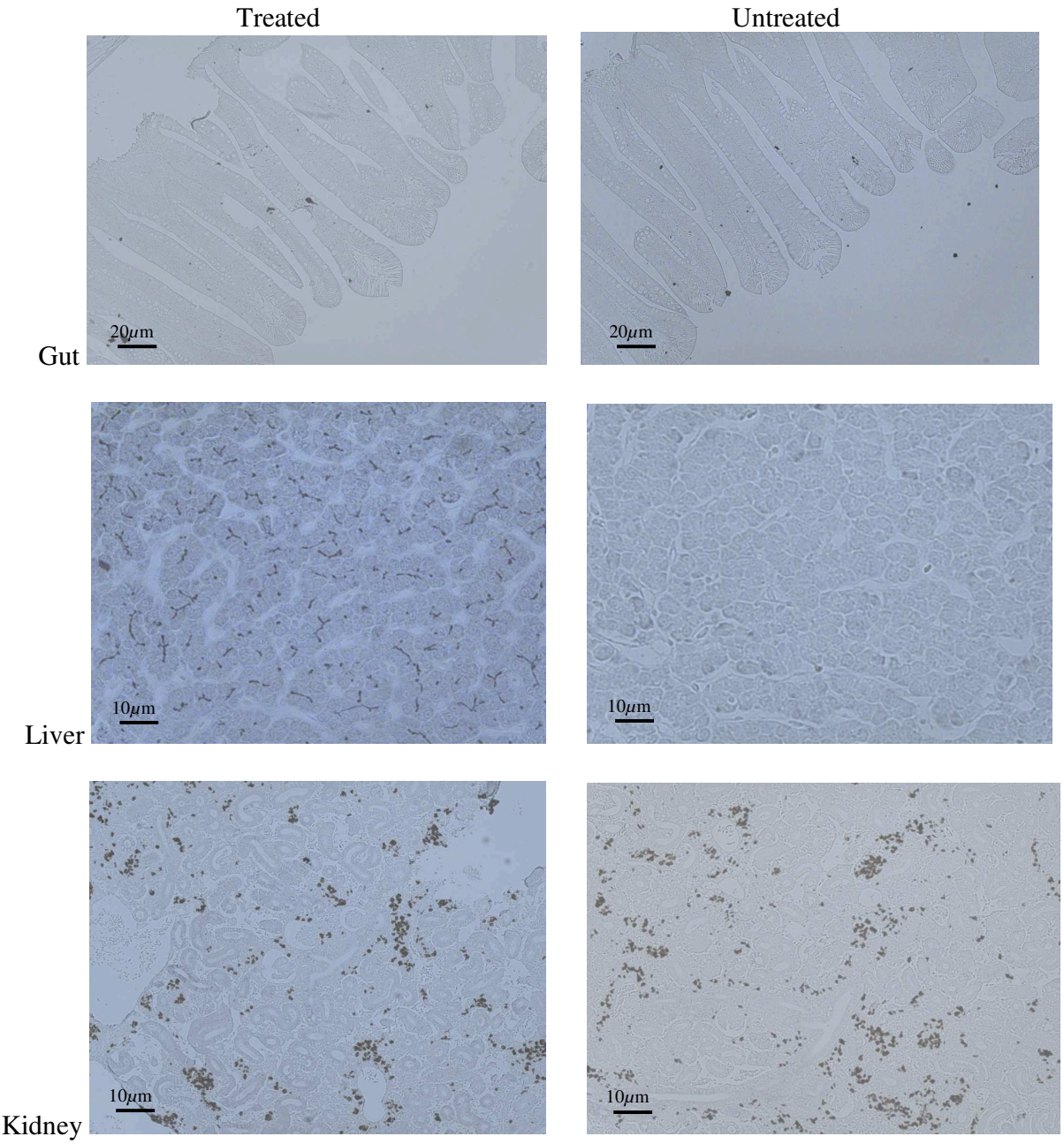
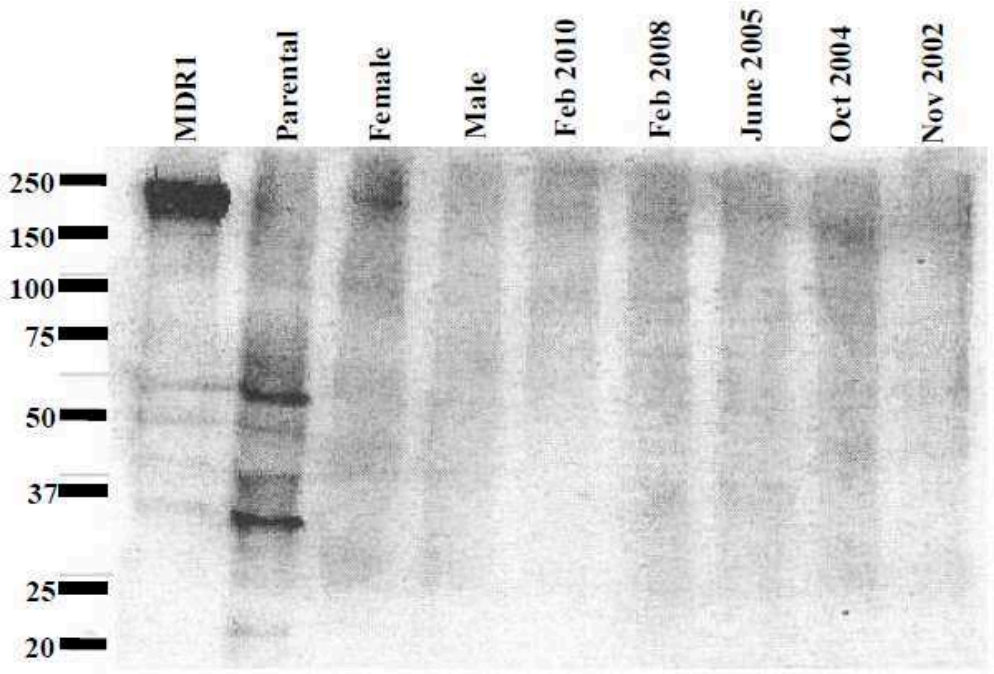
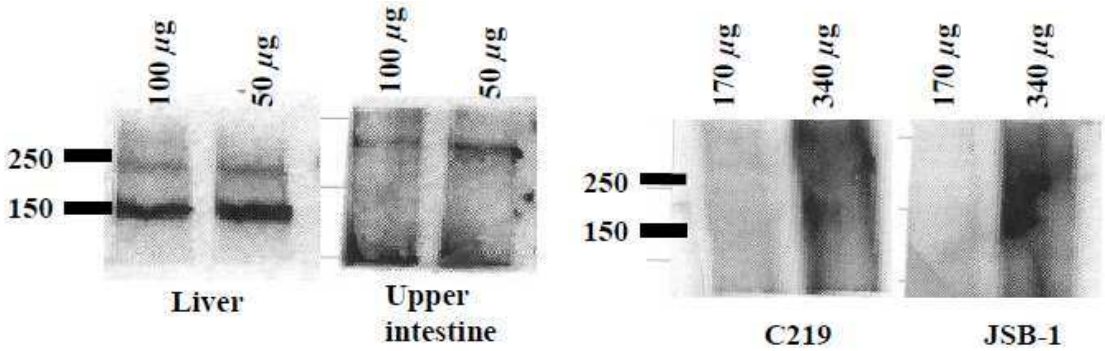


Figure 6.2. P-glycoprotein antibody (C219)-treated and -untreated *Salmo salar* tissues. Immunoreactions were detected only in the liver canaliculi (antibody-treated).



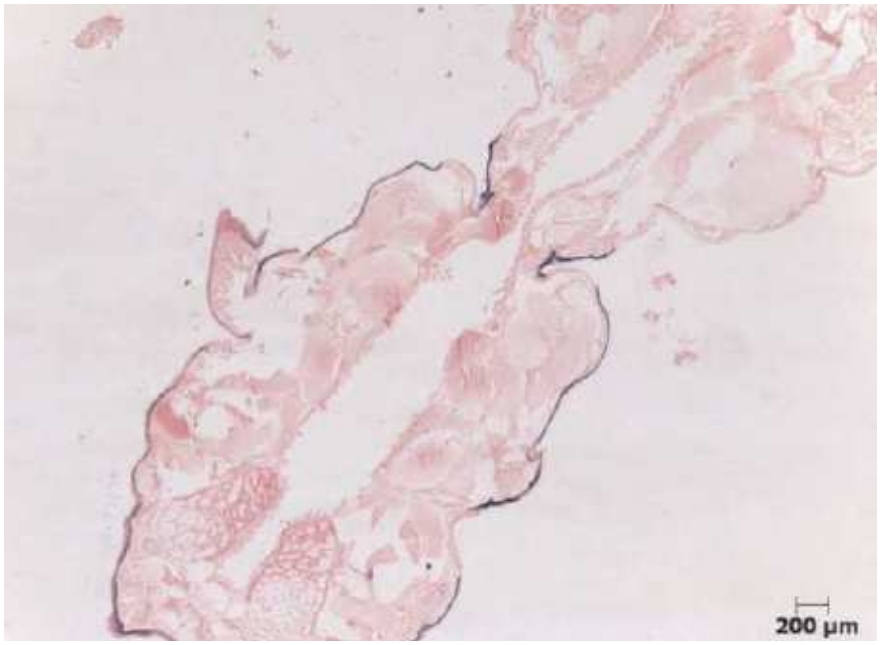
Sea lice and MDCK II cells



Salmon Lobster mid-intestine

Figure 6.3. Immunoblotting for P-glycoprotein in different populations of *Lepeophtheirus salmonis*, MDCK II cell lines, *Salmo salar* liver and upper intestine, and *Homarus americanus* mid-gut. C219 used except where stated otherwise. Protein ladder in KDa.

Antisense



Sense

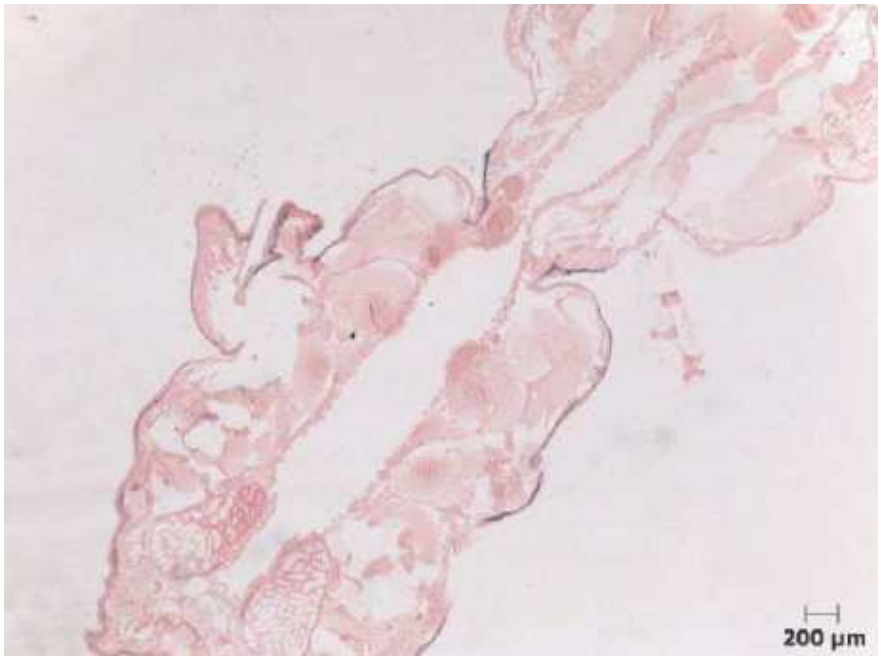


Figure 6.4. Detection of P-glycoprotein mRNA in *Lepeophtheirus salmonis* using *in situ* hybridization technique. No positive reactions observed.

Antisense

Sense

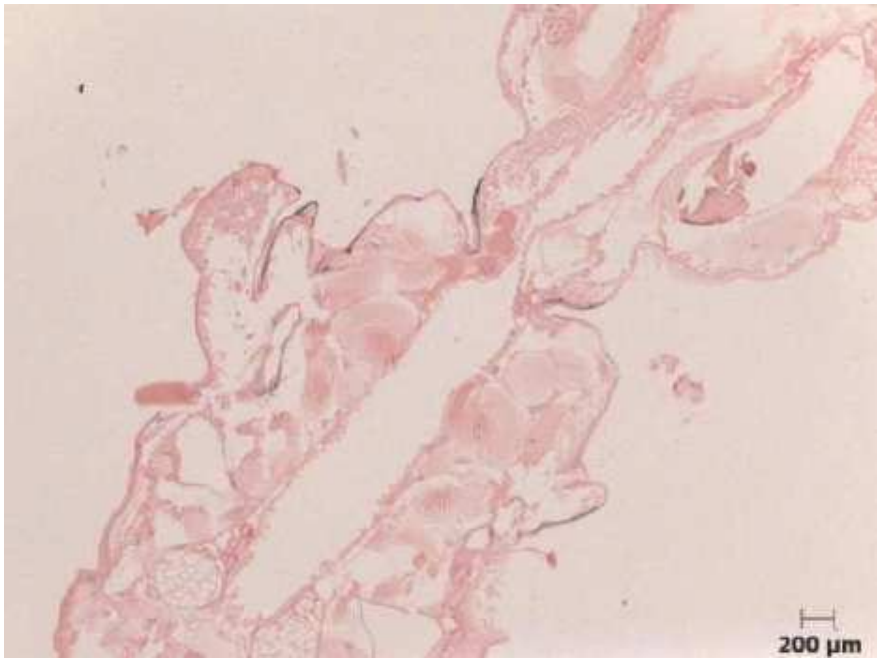


Figure 6.5. Detection of trypsin mRNA in *Lepeophtheirus salmonis* gut using *in situ* hybridization technique. Strong reaction was observed for the antisense RNA probe.

6.4.4. In situ hybridization

No reaction to P-gp RNA probes (sense and antisense) was detected for *L. salmonis* gut (Fig. 5.4); some reactions were observed at the cuticular matrix for the antisense probe. Reaction was detected in the gut of the parasite for the antisense trypsin positive control (Fig. 5.5).

6.5. Discussion

Immunodetection is commonly used to describe the abundance and distribution of proteins and mRNA of interest in a biological entity. The technique utilizes the ability of labeled antibodies or RNA probes to bind specific regions of a protein or mRNA, respectively, and has been employed for P-gp analyses in different tissues (van der Heyden et al. 1995). Unfortunately, we were neither able to detect P-gp mRNA and protein using *in situ* hybridization and immunolocalization, respectively, nor quantify the transporter using ELISA and Western blot techniques.

Our hypothesis was that P-gp mRNA is expressed in epithelial cells along the digestive tract of the parasite where the transporter causes the efflux of xenobiotics and lipophilic drugs including EMB (Tribble, Burka & Kibenge 2007). Several RNA probes targeting different regions of *L. salmonis* P-gp mRNA were used, but to no avail. This may be due to P-gp mRNA expression below minimum detection levels. Trypsin was detected in epithelial cells of *L. salmonis* digestive tract following the same tissue handling and processing employed for P-gp (Kvamme et al. 2004), and was successfully used as the positive control in the present study. Using an ELISA kit, *P-gp* was detected in mouse liver but poorly detected in sea lice, even when up to 500 μg total protein was used. The detection range of the ELISA kit for *P-gp* was 0.156-10.0 ng mL^{-1}. Based on the hypothesis that the transporter is expressed higher in the digestive tract of the parasite, pooled sea lice gut investigated for *P-gp* expression revealed levels below

the *P-gp* detection range of the kit. However, this does not conclusively imply low *P-gp* level in the pooled sea lice gut tissue examined. In addition, the ELISA kit was designed for detection of mouse *P-gp*, which may have resulted in less than optimal detection of *L. salmonis P-gp*. Thus, low expression and suboptimal antigenic specificity may have been the major reasons why detection of the transporter using Western blot and immunhistochemistry were unsuccessful. Studies have shown that even a single amino acid substitution in the epitope of a protein can lead to loss of antibody recognition (Georges et al. 1990). To overcome the putative obstacles to detecting and/or quantifying the transporter in *L. salmonis*, antibodies specific to the parasite *P-gp* need to be developed. In addition to that, techniques such as cryosectioning, which would minimize damage to *P-gp* epitope during tissue processing, have to be explored (Cordon-Cardo et al. 1990, Smith & Prichard 2002). It has been noted that formalin and paraffin treatment can potentially compromise the integrity of *P-gp* epitopes (Cordon-Cardo et al. 1990). Based on the positive controls utilized in this study, we believe that the unsuccessful detection of *L. salmonis* P-gp at the mRNA and protein levels was not as a result of poor methodology, tissue processing and handling. Also, whether there is more than one P-gp gene or isoform in *L. salmonis* is not known. Two different *P-gp* antibodies, C219 and JSB1, targeting different isoforms of the transporter were used in the present study, but yielded negative results for *L. salmonis*. A previous study identified different isoforms (classes I, II and III) of *P-gp* in hamster using C32, C219 and C494 monoclonal antibodies which target different *P-gp* epitopes (Georges et al. 1990). Nematodes have numerous P-gp genes compared with mammals (Sangster 1994, Broeks et al. 1995, Sangster 1999) with *C. elegans* possessing up to 15 (Prichard & Roulet 2007). *In situ* hybridization component of the present study was based on *L. salmonis* P-gp SL-PGY1 (GenBank accession number HQ684737) cloned by Heumann et al. (2012). More studies are

needed to develop *L. salmonis P-gp* antibody as it will be helpful in elucidating the expression pattern of the transporter at the protein. Also, more studies are necessary to determine whether there are other P-gp genes in *L. salmonis*.

6.6. References

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